STRAINS OF THE CROWN GALL PATHOGEN, *AGROBACTERIUM TUMEFACIENS*, AND THEIR BIOLOGICAL CONTROL IN SOUTH AFRICA

by

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DECLARATION

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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BIOGRAPHICAL SKETCH

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CONTENTS

INTRODUCTION

Crown gall, caused by *Agrobacterium tumefaciens*, is world-wide a disease of many crop plants, especially deciduous fruits and grapevines (Falk, 1977; Kerr, 1974; Schroth *et al.*, 1971; Stapp, 1961). The Gram-negative rod-shaped bacteria induce uncontrolled outgrowths of plant tissues, similar to animal cancers (Stapp, 1961).

Agrobacterium tumefaciens has been described as the plant pathogenic bacterium with the largest plant host range (Merlo, 1978). It is an obligate wound parasite, infecting plants through wounds caused in a variety of ways (Kerr, 1975; Kupila-Ahvenniemi, 1968). Only living *A. tumefaciens* cells can induce tumours (Veldstra, 1972). The main pathogenicity genes of *A. tumefaciens* are located on a large plasmid, the tumour-inducing or Ti-plasmid, within the bacterium (Kerr, 1978; Zaenen *et al.*, 1974). Tumour induction involves plant cell transformations, after which the presence of *A. tumefaciens* is no longer necessary for tumour proliferation. Tumours usually develop on the crown areas of infected plants, but can also occur on stems and shoots above ground, as on grapevines (Lehoczky, 1968). Crown gall tumours can be damaging to plants and can cause their death, specially when they become infected at a young age (Stapp, 1961).

Among plant diseases world-wide, crown gall is one of the most important economically and one of the best studied (Kerr, 1969a; Moore and Warren, 1979; Panagopoulos and Psallidas, 1973; Schroth *et al.*, 1971). In South Africa, it is a widespread disease of grapevines, deciduous fruit trees, ornamental plants, and other crops (Loubser, 1978) and is especially prominent in stone fruit and grapevine nurseries (Matthee *et al.*, 1979). According to information recorded by the Directorate of Plant and Liquor Control, Department of Agricultural Economics and Marketing, close to half a million plants were destroyed in South Africa from 1979 to 1988 following crown gall inspections of nurseries. This total excludes the plants destroyed by nurserymen prior to inspection and by farmers. For example, in the Upington area in 1976 approximately 2 000 ha of grapevines were destroyed due to high percentages of crown gall infections (J. Louw, South African Dried Fruit Board, Upington, personal communication).

Although plant inspectors and nurserymen are collaborating to prevent the sale of plants infected by *A. tumefaciens*, they can only eliminate plants showing visible crown gall symptoms. Apparently healthy plants may be latently infected and may develop tumours when replanted or during the next growth season, as experienced by Moore (1976) with *Prunus* spp. The sale of crown gall-infected plants is prohibited under the Agricultural Pest Act (Act 36 of 1983).

Among the many attempts to control crown gall by various treatments, the most promising have appeared to be those of Keane *et al.* (1970), and New and Kerr (1972) involving biological control with *Agrobacterium radiobacter* strain 84. This *A. radiobacter* strain produces agrocin 84, a bacteriocin active against *A. tumefaciens* (Kerr, 1974). When *A. radiobacter* strain 84 is applied to plant roots and crowns during transplanting, it effectively controls infection by *A. tumefaciens*. However, not all *A. tumefaciens* isolates are sensitive to agrocin 84. Reports of both successful (Kerr, 1974; Kerr and Panagopoulos, 1977; Lopez, 1978; Moore, 1979; Moore and Warren, 1979) and unsuccessful (Alconero, 1980; Grimm and Süle, 1981; López *et al.*, 1981; Scalza *et al.*, 1979) biological control have been published. Good control (approximately 98%) was achieved in a field trial with peach (*Prunus persica* cv. Du Plessis) in South Africa (Matthee *et al.*, 1977). However, Moore (1979) and C. G. Panagopoulos (Benaki Phytopathological Institute, Athens, Greece, personal communication) noted that there were various important factors involved in field biological control experiments, especially beneath the soil level, which could affect control by *A. radiobacter* strain 84.

Vastlanck Types.

Different biotypes of *A. tumefaciens* showing differences in certain cultural characteristics and in some cases host range, namely, biotypes 1, 2 (Keane *et al.*, 1970) and 3 (Kerr and Panagopoulos, 1977) have been identified in various countries, including South Africa (Loubser, 1978; Du Plessis *et al.*, 1984).

Serological differentiation of *Agrobacterium* strains has been correlated with differentiation based on other characteristics in only some of the studies. For example, there have been reports of serological differentiation (Riker *et al.*, 1930; Schilperoort *et al.*, 1969) or no

differentiation (Graham, 1971; Hochster and Cole, 1967) between pathogens and non-pathogens, and differentiation (Keane *et al.*, 1970) or no differentiation (Miller and Vruggink, 1981) between biotypes. Serological techniques can thus not be used indiscriminately for the rapid recognition of *A. tumefaciens* strains, but may have value if the necessary correlation between serotype and the characteristic of interest can be shown among the strains of a particular area. The new technique of enzyme-linked immunosorbent assay (ELISA) has shown promise for the recognition of serotypes of the related genus *Rhizobium* (Kishinevsky and Gurfel, 1980; Morley and Jones, 1980).

The taxonomy within the genus *Agrobacterium* was described by Kersters *et al.* (1973) as confusing and applicable only to the recognition and labelling of strains. Nonetheless, three species (*A. tumefaciens, Agrobacterium rhizogenes*, and *Agrobacterium rubi*) have been recognized on the basis of their pathogenic properties and one (*A. radiobacter*) on the basis of its non-pathogenicity (Buchanan and Gibbons, 1974; Krieg and Holt, 1984; Skerman *et al.*, 1980; Young *et al.* 1978a,b). However, an alternative grouping on the basis of modern taxonomic methods is into 3 biovars (biotypes) plus *A. rubi* (Krieg and Holt, 1984). Biotypes 1 and 2 occur in the present species *A. tumefaciens, A. radiobacter* and *A. rhizogenes*, and biotype 3 in *A. tumefaciens*. The proposal (Krieg and Holt, 1984) that the biovars should be made the basis of the species, is blocked at present by Opinion 33 of the Judicial Commission (1970). In spite of the preference for the term 'biovar' (Krieg and Holt, 1984) the established term 'biotype' rather than 'biovar' is used in this thesis.

The steady increase in the incidence and severity of crown gall in South African nurseries, orchards and vineyards in recent years prompted the present study of the pathology and biology of the crown gall organism. The availibility of data on the crown gall disease and its total effect in South Africa is limited. Little is known about the ecology of *A. tumefaciens* or its distribution and abundance in nurseries, orchards and vineyards throughout the country. The following studies were therefore undertaken:

(i) Development of effective procedures to isolate *A. tumefaciens* from infected plant material and soils in South Africa.

(ii) Development of procedures for the inoculation of isolated *A. tumefaciens* on suitable indicator plants, to produce tumours non-selectively in the shortest possible time.

(iii) Determination of the host plants of South African *A. tumefaciens* strains, as well as the geographical distribution of the strains.

(iv) Biotyping of South African *A. tumefaciens* isolates according to the method described by Kerr and Panagopoulos (1977). This was to be a more extensive study than those of South African biotypes and genetic groups conducted by Loubser (1978) and Du Plessis *et al.* (1984).

(v) Investigation of whether serological techniques would provide reliable and rapid methods of pathogen identification. Although the crown gall disease is widespread, there is great concern about its continuing spread, and much interest in its early detection. As pathogenicity studies on suitable host plants according to Koch's postulates and the biotyping of isolates are very time consuming, it is essential that fast, reliable and uncomplicated detection methods for the crown gall bacteria be developed. Thus, the possibility of serological differentiation of pathogens and non-pathogens, as well as of different biotypes, was examined. Serotyping was undertaken to determine the heterogeneity of South African *A. tumefaciens* isolates and whether serotypes could be grouped according to geographical areas or host plants or both. Serological comparisons were also made between local and overseas *A. tumefaciens* isolates.

(vi) Studies of the *in vitro* inhibition of South African *A. tumefaciens* isolates by the bacteriocinogenic strains *A. radiobacter* K84 and *A. tumefaciens* D286 (a local strain), on three different media.

(vii) Investigations of *in vivo* inhibition of crown gall formation by the use of the bacteriocinogenic strains mentioned under (vi) to suppress South African pathogenic *A. tumefaciens* isolates. In these experiments, which were conducted in a glasshouse with two different indicator plants, the bacteriocinogenic strains and the pathogenic isolates were applied separately and together in different ratios on tobacco and datura plants. The effects of different times of inoculation of the two bacteriocinogenic strains in relation to inoculation of the pathogens were also investigated.

(viii) Evaluation of several possible chemical and physical crown gall control measures.

LITERATURE REVIEW

Discovery and Naming of the Crown Gall Pathogen

Knobby outgrowths (galls) on the crown and other parts of plants have been described since the earliest times. The cause of the galls was first attributed to bacteria by Italian botanists in 1880-1890 (Gram and Weber, 1953). Later Smith and Townsend (1907) described the crown gall disease and were the first to isolate the pathogen, a Gram-negative rod-shaped bacterium which they subsequently named *Bacillus tumefaciens*, from *Chrysanthemum frutescens* (Paris daisy). When this bacterial isolate, as a pure culture, was inoculated onto the original host plant species, typical crown gall symptoms appeared.

Hereafter, the crown gall organism was also known under the names *Bacterium tumefaciens*, *Pseudomonas tumefaciens* and *Polymonas tumefaciens* (Buchanan and Gibbons, 1974). These names were not accepted but on the basis of additional tests and a different classification system, became *Agrobacterium tumefaciens* (Conn, 1942). The genus *Agrobacterium* was placed in the family Rhizobiaceae.

Crown gall is a cancerous disease of plants with similarities to animal cancers (Kupila-Ahvenniemi, 1968). The disease may also be induced by species of *Agrobacterium* other than *A. tumefaciens* (Kerr, 1975).

Economic Importance of Crown Gall

In most countries where the crown gall pathogen, *A. tumefaciens,* occurs, the disease seems to be of economic importance (Abo-El-Dahab *et al.*, 1978; Kerr, 1974; Király *et al.*, 1970; Matthee *et al.*, 1977; Panagopoulos *et al.*, 1978, 1979; Stapp, 1961; Taha *et al.*, 1975).

Crown gall is a common disease of deciduous fruit trees, grapevines, and other crops in South Africa (Loubser, 1978), and is especially prominent in stonefruit nurseries (Matthee *et al.*,

1979). In Europe and North America, apples, pears, grapevines and other crops have been seriously affected by this disease and in Australia, stonefruit and roses (Schroth *et al.*, 1971). Schroth *et al.* (1971) stated that the annual loss as a result of crown gall infections in California alone exceeded $7 000 000. Economic losses in nurseries occurred because the U.S. Government prohibited the interstate shipments of diseased plants, and ordered these plants destroyed (Moore and Warren, 1979). Plants devoid of visual symptoms are sold, but the dangers of latent infections and infested soil surrounding plant roots have to be considered (Kerr, 1969a). Epidemic outbreaks of 80-100% infection have been recorded on some nursery material (Moore and Warren, 1979).

Losses are not restricted to nurseries alone, but can also pose a serious problem in orchards and landscape plantations (Moore and Warren, 1979). Crown gall can destroy grapevines (Erasmus *et al.*, 1974; Loubser, 1978), hops and immature stonefruit trees (Dhanvantari, 1976; Kerr, 1969a; Király *et al.*, 1970; Panagopoulos and Psallidas, 1973; Schroth and Moller, 1976), poplars and willows (Spiers, 1979). The disease pattern may fluctuate from year to year (Kerr, 1969a; Moore and Warren, 1979).

Geographic Distribution and Host Range of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a common pathogen in Africa, Asia, Australia, Europe, and North and South America (Abo-El-Dahab *et al.*, 1978; Du Plessis *et al.*, 1984; Falk, 1977; Kerr, 1969a, 1980; López *et al.*, 1981; Loubser, 1978; Matthee *et al.*, 1979; Panagopoulos and Psallidas, 1973; Robbs *et al.*, 1981; Schroth and Moller, 1976; Spiers, 1979). As the bacteria have such a wide geographical range, many countries have become concerned about the risk of the crown gall disease, and it is therefore essential that reliable and uncomplicated identification methods be developed (Miller and Vruggink, 1981). *Agrobacterium tumefaciens* can be dispersed by irrigation water leading to the spread of crown gall disease in irrigated agricultural areas (Smith and Cochran, 1944).

Agrobacterium tumefaciens has been described as the plant pathogen with the widest host range (Merlo, 1978). Braun (1959) noted that it infected plants of 142 genera in 61 different botanical families, but more recent studies have extended its host range to at least 640 different plant species in 93 different families of dicotyledonous and gymnospermous plants (Krieg and Holt, 1984). Dicotyledonous plants in particular are infected (Braun, 1959), mostly in the crown, root, stem and shoot regions (Stapp, 1961). According to Kupila-Ahvenniemi (1968), *A. tumefaciens* includes strains that differ in their virulence to different plant species, and strains that are pathogenic for a certain host or hosts but unable to induce tumours on other potential hosts. According to Stapp (1961), *A. tumefaciens* strains cannot be classified according to the plants which they infect, except possibly the strains that infect grapevines.

Mechanisms of Plant Infection and Tumour Induction by *Agrobacterium tumefaciens*

Moore and Tingey (1976) observed that damage caused by crown gall is greatest in young plants. Prevention of wounds in early stages of plant development is therefore very important. Moore (1976) also observed that cool soil or low air temperatures can extend wound healing and the period of susceptibility to *A. tumefaciens* infection.

Only living *A. tumefaciens* cells can induce tumour formation (Veldstra, 1972). Wounding is essential for infection to occur (Kerr, 1975; Kupila-Ahvenniemi, 1968), but the method of wounding is unimportant (Kupila-Ahvenniemi, 1968). Although basically all living nucleated plant cells, except lignified cells, can be transformed to tumour cells, they must first be conditioned by the stimulus of a wound to be susceptible for the pathogen (Braun, 1959). The dimensions of the tumour depend on the size of the wound (Kupila-Ahvenniemi, 1968; Rogler, 1981). Usually the pathogen becomes localized in the intercellular spaces of the infected plant tissue (Bogers, 1972). Chemotaxis may be a significant initial reaction as a strong correlation between possession of a Ti-plasmid and chemotaxis was established by Ashby *et al.* (1988). They discovered this movement by using virulence-inducing phenolic compounds as chemoattractants. Positive chemotaxis toward root and shoot homogenates from

monocotyledonous and dicotyledonous plants was observed, and at low extract concentrations, chemotaxis was enhanced by the presence of the Ti-plasmid within the pathogen.

Besides supplying a portal of entry for the bacteria, the wound bares specific binding sites of plant cells to complementary binding sites on the pathogen (Lippincott and Lippincott, 1977, 1980). Binding is indicated by electron micrographs showing the pathogen closely associated with the plant cell walls (Bogers, 1972; Smith and Hindley, 1978). Scanning electron micrographs showed non-pathogenic *Agrobacterium* strains to be loosely and disorderly arranged around tobacco cells (Matthyse *et al.*, 1978; Smith and Hindley, 1978). Cells of pathogenic strains, however, clumped together, and whole plant cell surfaces were sometimes covered with bacteria attached polarly (Matthyse *et al.*, 1978; Smith and Hindley, 1978) or laterally (Matthyse *et al.*, 1978) to the cell walls. Lippincott *et al.* (1977) showed that plant cell walls contained surfaces to which virulent *A. tumefaciens* cells could attach. They proposed that the specificity of the host-*A. tumefaciens* interaction was determined by this attachment. Pretreatment of wound areas with inactivated site-specific bacteria inhibited tumour induction by virulent strains. Cells of non-site-specific agrobacteria had no such inhibitory effect. Plant cell membrane fractions showed no specific binding properties in regard to the pathogens.

Pectin seems to be an important component involved in the binding reaction (Lippincott and Lippincott, 1969; Lippincot *et al.*, 1977; Pueppke and Benny, 1983). The pectin of crown gall-susceptible dicotyledonous plant cells differs from that of non-susceptible monocotyledonous or crown gall tumour cells in respect of the amount of methyl esterification of the pectin (Sequeira, 1978). The pathogen binds onto the unesterified galacturonic acid residues of dicotyledonous plant cells. The galacturonic acid residues of monocotyledonous plants are esterified to such an extent that binding of *A. tumefaciens* cells is impossible. Polygalacturonic acid residues from Pinto bean leaves were an excellent inhibitor of *A. tumefaciens* infection. Complete inhibition was obtained by 10 mg/ml and 50% inhibition at 1 μg/ml polygalacturonic acid. So far no plant lectin involvement has been reported. The walls of cells of crown gall tumours and embryonic plant tissue contain no *Agrobacterium* binding sites (Tate *et al.*, 1979).

Although Manasse and Corpe (1967) found similar carbohydrates in the cell envelopes of both virulent and avirulent *Agrobacterium* strains, it seems from more recent studies (Lippincott and Lippincott, 1980; Matthyse *et al.*, 1978; Sequeira, 1978; Whatley *et al.*, 1978) that the bacterial lipopolysaccharide (LPS) may be the major component involved in the attachment of the bacteria to wound cells. Whatley *et al.* (1978) found that *Agrobacterium* cell wall preparations, Boivin antigen and surface LPS from the bacteria were effective tumour inhibitors when added to plant wounds before or together with the pathogen. Preparations of LPS from *A. radiobacter* and *A. tumefaciens* strains that did not attach to plant cell walls, did not appear to be successful inhibitors of tumour formation (Matthyse *et al.*, 1978; Whatley *et al.*, 1978). Sequeira (1978) showed that the LPS preparations had the same binding specificity as intact bacterial cells. The wound site binding specificity of *A. tumefaciens* strains explains the competitive inhibition of tumour formation by *A. tumefaciens*, but not by *Escherichia coli* or *Rhizobium leguminosarum*, as observed by Bogers (1972). The *E. coli* and *R. leguminosarum* also showed no binding to plant cells (Bogers, 1972). Whatley *et al.* (1978) postulated that the O-antigenic external projecting chains of the LPS were involved in a conformation type binding mechanism with the polygalacturonic acid units of the pectin of the plant cell walls exposed by the wounding process. The involvement of pectin in the binding reaction is possibly the reason for the wide host range of the crown gall pathogen.

After the initial binding of virulent *A. tumefaciens* strains to plant cells, cellulose fibrils are produced by the pathogen whereby it anchors its cells to the plant cell walls (Loper and Kado, 1979; Matthyse *et al.*, 1981). Through their catabolism of pectin the *Agrobacterium* cells come into contact with the host cell plasma (Loper and Kado, 1979).

It was discovered by Tarbah and Goodman (1988a) that the xylem parenchyma was more sensitive to transformation and tumour formation than other plant cell types. Most cell divisions occurred in the upper layer of the inoculated cut surface of the plant stem segments. The pathogen appeared to multiply primarily in the xylem vessels for the first 48 h. About 48-60 h after inoculation the bacterial numbers in the vessels were greatly diminished. Other observations by Tarbah and Goodman (1988b) showed that *A. tumefaciens* became

pleomorphic and formed inclusions that migrated to the polar positions of the bacterial cell. From these positions these inclusions were discharged into the areas of the xylem vessel lumen. The pleomorphism and inclusions that preceded lysis suggest that the lytic process provides a manner in which the T-DNA might be released. This lysis also appeared to be the cause of the reduction of large numbers of *A. tumefaciens* in xylem vessels 48-60 h after inoculation.

After completion of the infection process, the growth of tumours is independent of the presence of the causative bacteria (Braun, 1959; Kado, 1976; Veldstra, 1972). The cells of certain plant species have high regenerative activity and instead of characteristic crown gall tumours, teratomas are formed. The teratoma is a complex growth of highly abnormal leaves and buds in different stages of morphological development.

The severity of the crown gall disease is dependent on temperature, the portal of infection and plant age (Moore and Tingey, 1976). The largest growth reduction from crown gall was temperature-dependent and occurred at the same temperatures that maximized top growth of non-infected control plants. Significant differences in plant growth occurred when various sites on plants were infected, but growth reduction was most severe when younger plants where inoculated, regardless of the infection site (Moore and Tingey, 1976; Stapp, 1961). Total dry weight reduction was greatest when the upper hypocotyl was infected. Young plants infected in the upper hypocotyl were permanently stunted, and failed to recover after an extended growth period. Infected plants were also in a generally weaker condition than healthy plants (Kiraly *et al.*, 1970). Large tumours on mature plant roots are more detrimental to plant growth than tumours on the crown area which result in relatively normal plants (Gram and Weber, 1953). The tumours on herbaceous plants consist mainly of soft parenchymatic tissue whereas tumours on woody plants are initially soft but later become woody and hard. This hard, woody tissue consists mainly of tracheids (Gram and Weber, 1953; Kiraly *et al.*, 1970; Stapp, 1961).

In natural infections it is mainly the crown and root areas that show symptoms (Gram and Weber, 1953; Kiraly *et al.*, 1970; Taha *et al.*, 1975). Natural wounding which provides portals

for infection can be caused in a variety of ways, for example, by friction, freezing and thawing (Kupila-Ahvenniemi, 1968), and under laboratory conditions any plant part can be infected (Gram and Weber, 1953; Kiraly *et al.*, 1970). The pathogen can spread systemically through the xylem tissue, and cause secondary tumours, as was shown on grapevine (Katz and Burr, 1982; Lehoczky, 1968, 1978) and *Datura tatula* (syn. *Datura stramonium*) (Stapp, 1961).

Host cells in infected tissue are not destroyed, but are stimulated to divide in an uncontrolled and uneven manner (Stapp, 1961). Normal plant cells can be transformed into tumour cells only during a limited time period in the normal wound healing process (Braun, 1959, 1962; Veldstra, 1972), the optimum time being about 60 h after wounding (Kupila-Ahvenniemi, 1968). The unregulated growth of the tumour causes disorganization of the tissue, hypertrophy, hyperplasia and cytological deviation of the cells. Proliferating tumour cells are no longer callous tissue, as they do not show the reactions of normal callous tissue. Tumours produce new cell types with newly acquired characteristics, the most important being the capacity for continuous unregulated growth in the absence of any infective agent (Braun, 1959; Gram and Weber, 1953). The morphology of unorganized tumours could represent a type of wound healing that starts in a normal way, but where the gene responsible for healing becomes "locked on" (Braun, 1959; Merlo, 1978). The transfer of agrobacterial DNA into plant cells thus causes these cells to remain in a fast-growing active and dividing condition (Merlo, 1978).

Certain unusual L-amino acids, the opines, have been found only in crown gall tumours (Drummond, 1979; Petit *et al.*, 1978; Schell, 1978). The type of opine produced depends on the nature of the infecting bacterial strain. The tumour opines are nopaline, octopine and agropine. Nopaline has been identified as N^2-(1,3-D-dicarboxypropyl)-L-arginine (Goldmann *et al.*, 1969) and octopine as N^2-(1-D-carboxyethyl)-L-arginine (Ménagé and Morel, 1964). Agropine is a lactone, formed *in vivo* from N^2-(1-D-mannityl)-L-glutamine (mannopine) (Tempé and Goldmann, 1982). Infective *A. tumefaciens* strains can use the opines as sole carbon and nitrogen sources and only these strains can survive inside the tumours (Brill, 1981; Drummond, 1979). This selectivity is an important factor determining host-pathogen specificity. *Agrobacterium tumefaciens* strains which catabolize octopine induce octopine-

synthesizing tumours, whereas strains catabolizing nopaline induce nopaline-synthesizing tumours. The same principle applies to agropine tumours. Certain strains of *A. tumefaciens* can utilize both octopine and nopaline (Kerr and Roberts, 1976). Strains unable to catabolize either octopine or nopaline became known as null type organisms causing null type tumours (Kerr and Roberts, 1976; Merlo, 1978; Montoya *et al.*, 1977); however, null type tumours not containing octopine or nopaline were found to produce agropine (Firmin and Fenwick, 1978). Most octopine-containing tumours also contain agropine (Drummond, 1979; Sykes *et al.*, 1981). Köhn and Beiderbeck (1982) have found that certain *Pseudomonas* strains can also utilize octopine.

Kerr (1978) and Zaenan *et al.* (1974) found that all tumorigenic strains of *A. tumefaciens* contained a large circular extrachromosomal plasmid, the Ti-plasmid. This plasmid contains the genes coding for virulence, the catabolism of octopine (Chilton *et al.*, 1976; Van Larebeke *et al.*, 1975; Watson *et al.*, 1975) or nopaline (Watson *et al.*, 1975), and the synthesis of these opines in tumours (Bomhoff *et al.*, 1976). After bacterial attachment to a host cell, the T-DNA, a part of the Ti-plasmid, is transferred to the host cell where it is incorporated into the plant DNA and subsequently expressed (Merlo, 1978; Zambryski *et al.*, 1980). The incorporated T-DNA can be replicated in progeny cells of the transformed cell (Zambryski *et al.*, 1980). No natural barrier exists between the procaryote and the eucaryote in respect of this transfer of genetic material (Drummond *et al.*, 1977). Loss of the Ti-plasmid by an *A. tumefaciens* strain results in the loss of pathogenicity, and if a Ti-plasmid is transferred to a non-infective strain, it develops the ability to induce crown gall tumours (Kerr, 1971).

Actually there are different Ti-plasmids (Thomashow *et al.*, 1981), two commonly found types being the octopine- and nopaline Ti-plasmids (Currier and Nester, 1976). The octopine Ti-plasmid codes for pathogenicity and octopine metabolism (Montoya *et al.*, 1977), whereas the nopaline Ti-plasmid codes for pathogenicity, nopaline metabolism, sensitivity to agrocin 84 and conjugation (Kerr, 1978).

The Ti-plasmids are conjugative (Genetello *et al.*, 1977) and can apparently mobilize otherwise non-transferable plasmids (Kerr, 1978). The transfer genes of both Ti- and non-transferable plasmids are usually completely suppressed, but are derepressed by octopine or nopaline that serve as aphrodisiacs and promote agrobacterial conjugation (Kerr, 1978). The Ti-plasmid, through its effect on octopine or nopaline synthesis in tumours, thus promotes not only the selective development of its host bacterium but also its own transfer through the bacterial population in the tumour (Brill, 1981; Drummond, 1979).

The Ti-plasmid also codes for the host range of the pathogen (Loper and Kado, 1979; Sonoki *et al.*, 1978; Thomashow *et al.*, 1980; Watson *et al.*, 1975). Matthyse *et al.* (1978) observed higher percentages of attachment to host cells by bacteria containing the Ti-plasmid than by bacteria not containing the Ti-plasmid. However, the Ti-plasmid was not the only genetic structure controlling binding, as an avirulent Ti-plasmidless *A. tumefaciens* strain, NT1, also attached to tobacco cells. The LPS of this strain inhibited attachment of virulent strains to the plant cells.

Taxonomy of the Genus *Agrobacterium*

In the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974), *Agrobacterium* species resort under the family Rhizobiaceae in Part 7, Gram-negative aerobic rods and cocci. All *Agrobacterium* species except *Agrobacterium radiobacter* induce cortical hypertrophies on roots and stems of different plant species. *Agrobacterium* species are rod-shaped bacteria (0.8 x 1.5-3.0 um), produce no endospores, are motile by 1-4 peritrichous flagella, grow well on carbohydrate-containing media, and excrete copious amounts of extracellular slime on these media. The organisms are oxidase-positive, have a respiratory metabolism without gas production, and although aerobic, can live under reduced oxygen concentrations in plant tissue. A serum zone which enlarges gradually, is formed in litmus milk. *Agrobacterium tumefaciens* and *A. radiobacter* produce 3-ketolactose from lactose. *Agrobacterium tumefaciens* cells show star formations in a carbon rich and nitrogen poor medium (Stapp, 1961).

In Volume 1 of Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984), the genus *Agrobacterium* resorts under Family III, Rhizobiaceae in Section 4, Gram-negative aerobic rods and cocci. The description of *Agrobacterium* in this manual is very similar to that in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) with a few additions, for example, that some strains possess a wide host range, whereas others, such as grapevine isolates, possess a limited host range. Tumour induction by *Agrobacterium* is correlated with the presence of a large tumour-inducing plasmid (Ti-plasmid) in the bacterial cells. Biochemical reactions, such as changes in litmus milk and 3-ketolactose production from lactose, distinguish biotype 1 from biotype 2 strains. One group of biotype 1 strains produces an alkaline reaction in litmus milk, usually accompanied by the formation of a brown, clear serum zone. Strains of biotype 2 produce an acid reaction causing this medium to become pink. The rapid and unusual oxidation of lactose to 3-ketolactose by biotype 1 is an important biochemical test for its rapid differentiation from the 3-ketolactose-negative biotype 2 and 3 strains. Opines (octopine and nopaline) are unusual amino acid derivatives produced in tumour tissues following induction by oncogenic strains of *Agrobacterium*. At least three genetic groups of Ti-plasmids can be recognized in wild type *Agrobacterium* strains according to the type of opine that is synthesized by the tumour and utilized by the bacterium. These groups are:

(a) The octopine-type Ti-plasmids, which are highly homogeneous.
(b) The nopaline-type Ti-plasmids, which are a more diverse group.
(c) The agropine-type Ti-plasmids, formerly known as the null-type Ti-plasmids, which induce the production of agropine but not of octopine or nopaline, in tumours.

The following functions are known to be determined by the Ti-plasmids:

(a) Oncogenicity.
(b) The nature of the opine(s) synthesized in the transformed plant cells.
(c) The utilization of opines and arginine.

(d) The conjugative transfer of the Ti-plasmid (promoted by the opines).

(e) Sensitivity to the bacteriocin agrocin 84.

(f) The host range.

(g) The exclusion of bacteriophage AP-1.

The best known bacteriocin from *Agrobacterium* is agrocin 84, synthesized by the non-oncogenic *A. radiobacter* strain 84. Serological studies have indicated that it is impossible to distinguish oncogenic from non-oncogenic agrobacteria, but strains belonging to biotypes 1 and 2 may be distinguished from each other by serological reactions. The host range of *A. tumefaciens* is very wide and includes various families of dicotyledonous and gymnospermous plants. However, none of 250 monocotyledonous species investigated was susceptible to the disease. Three selective media have been described for the isolation of biotype 1, 2 and 3 strains from soil and crown gall tissues.

Colonies of *Agrobacterium* species on nutrient agar are usually small (1-4 mm), circular, convex, shiny and non-pigmented (Buchanan and Gibbons, 1974; Butler and Jones, 1949; Kiraly *et al.*, 1970; Krieg and Holt, 1984; Stapp, 1961).

Agrobacterium species have long been classified according to the host plants that they infect and the types of symptoms that they produce (Buchanan and Gibbons, 1974). Tissue-damaging inoculation tests on suitable indicator plants are necessary to confirm the identification of strains (Buchanan and Gibbons, 1974; Lehoczky, 1968; Schroth *et al.*, 1965). The key for the recognition of the species of *Agrobacterium* as given in the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) is as follows:

Amino acids, nitrates and ammonium salts utilized as an only nitrogen source; 3-ketolactose produced.

Produces tumours.

A. tumefaciens

Does not produce tumours.

A. radiobacter

Do not utilize amino acids, nitrates and ammonium salts as sole carbon source; 3-ketolactose not produced.

Produces hairy root of nursery stock.

A. rhizogenes

Produces galls on raspberry.

A. rubi

Eleven other *Agrobacterium* species were listed under *species insertae sedis*.

The same *Agrobacterium* species are listed in the new Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) but with considerable argument for a new approach to species recognition in the genus (Kersters and De Ley, 1984) This approach is only one of several attempts by *Agrobacterium* taxonomists during the past two decades to improve the pathogenicity-based classification of the 8th edition of Bergey's Manual (Buchanan and Gibbons, 1974).

White (1972), following a numerical taxonomy study involving strains of *Agrobacterium* and *Rhizobium*, proposed consolidation of the two genera under the genus *Rhizobium* with recognition of the following four groups of fast growing organisms:

Group I. *Rhizobium radiobacter*. A species containing both saprophytic, tumorigenic and a few hairy root-producing organisms. The ability to form 3-ketolactose, characteristic of biotype 1 agrobacteria (Bernaerts and De Ley, 1963), seemed to be characteristic of this species.

Group II. *Rhizobium meliloti*. The position of *A. rubi* in relation to this group was considered, but White (1972) concluded that the taxonomy of strains named *A. rubi* needed further clarification. The true position of *A. rubi* H36 is dubious since it failed to cluster with this group.

Group III. *Rhizobium rhizogenes*. Crown-gall- and hairy root-forming organisms as in Group I. The biotype 2 *Agrobacterium* strains of Keane *et al.* (1970) clustered in this group. Group III, however, was clearly separated from Group I and the *Rhizobium meliloti* cluster.

Group IV. *Rhizobium leguminosarum*. Strains of *R. leguminosarum*, *Rhizobium trifolii* and *Rhizobium phaseoli* were included in this species.

Crown-gall organisms were associated with groups I and III. The proposed *Rhizobium* species were defined without reference to pathogenicity or nodulating ability and it appears that crown-gall organisms can be distinguished only by pathogenicity testing. White (1972) proposed that the group I bacteria, comprising saprophytic *A. radiobacter*, 3-ketolactose-positive tumorigenic bacteria (*A. tumefaciens*) and the 3-ketolactose-positive pathogenic strains causing hairy root (*A. rhizogenes*) were the same and should be called *Rhizobium radiobacter*. These strains could be separated into subgroups by their symptoms or lack thereof on test plants. The name *Rhizobium rhizogenes* was proposed for the group III bacteria, comprising pathogenic strains previously designated *A. rhizogenes* and *A. tumefaciens*.

Holmes and Roberts (1981) also subjected *Agrobacterium* to numerical taxonomic analysis and separated the genus into four clusters. For the two major clusters, regardless of the phytopathogenic effect of the strains, the names *A. tumefaciens* and *A. rhizogenes* were used. The third cluster was mainly *A. rubi* and the fourth a yellow pigmented group. The species name *A. radiobacter* was rejected as this name was synonymous with *A. tumefaciens*, which being the type species, obviously had priority. Phytopathogenicity of strains within the species could be indicated by the terms saprophytic, tumorigenic and rhizogenic (Holmes and Roberts, 1981; Keane *et al.*, 1970; White, 1972).

The results of the numerical taxonomy studies of Keane *et al.* (1970) and Kersters *et al.* (1973) correlated with those of White (1972) and Holmes and Roberts (1981) in showing that strains of *Agrobacterium* clustered in three major groups. Notwithstanding the clustering shown in their numerical taxonomic analysis, Keane *et al.* (1970) concluded that *A. radiobacter, A. tumefaciens, A. rhizogenes* and *A. rubi* should be reclassified as one species and according to the International Code of Nomenclature of Bacteria and Viruses (Editorial Board of the International Commitee on Bacterial Nomenclature, 1958), the name should be *A. radiobacter* (Keane *et al.*, 1970). However, they noted that strains which had been designated *A. tumefaciens*, *A. rhizogenes* and *A. rubi* could be distinguished by their pathogenicity and host range, and that the pathogenicity of *A. rubi* was not restricted only to *Rubus* species. They

therefore proposed that all *Agrobacterium* cultures could be defined by a varietal epithet indicating pathogenicity and information on the biotype. The following classification and naming of *Agrobacterium* varieties was proposed by Keane *et al.* (1970):

(a) *A. radiobacter* var. *radiobacter* - non-tumorigenic

(b) *A. radiobacter* var. *tumefaciens* - tumorigenic

(c) *A. radiobacter* var. *rhizogenes* - hairy root

Agrobacterium rubi could not be classified as a variety of *A. radiobacter*.

Earlier results of numerical taxonomy (Graham, 1964; Moffet and Colwell, 1969) and DNA hybridization studies (Heberlein *et al.*, 1967) also indicated that the crown gall pathogen, *A. tumefaciens*, could not be distinguished from the saprophytic species, *A. radiobacter*. Kerr (1969b) had concluded that pathogenicity was not a satisfactory criterion for distinguishing species as *A. radiobacter* could be converted to *A. tumefaciens* by transfer of virulence genes.

Keane *et al.* (1970) also made provision in their classification for the indication of biotypes after the variety name if desired. Typical strains of *A. tumefaciens* as described in the then current 7th edition of Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957) were designated *A. radiobacter* var. *tumefaciens* biotype 1. Atypical crown gall organisms described by Kerr (1969a) were named *A. radiobacter* var. *tumefaciens* biotype 2.

Moore *et al.* (1980) commented that the nomenclature of Keane *et al.* (1970) showed high degrees of relationship but was cumbersome; furthermore, the diagnostic characters separating the biotype 1 and 2 organisms were sufficient to make them separate species.

Agrobacterium pseudotsugae, Agrobacterium gypsophilae, and *Agrobacterium stellulatum* should not be included in the genus *Agrobacterium* (De Ley *et al.*, 1966; Heberlein *et al.*, 1967; White, 1972). De Ley (1968, 1978) and White (1972) proposed that *A. gypsophilae* be reclassified as *Enterobacter agglomerans* (syn. *Erwinia herbicola*), and that *A. pseudotsugae* was a coryneform organism (according to DNA:rRNA hybridization tests).

Kersters *et al.* (1973) supported the criticism of Keane *et al.* (1970) of classification of the genus *Agrobacterium* into species according to pathogenicity on the grounds that it did not reflect natural relationships among these bacteria. No correlation existed between the traditional nomenclature (Breed *et al.*, 1957), and the real taxonomic structure as revealed by modern molecular and computer-assisted techniques.

In their more recent discussion in Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984), Kersters and De Ley (1984) proposed that the best solution for the taxonomy and nomenclature of *Agrobacterium* would be to give a separate species or subspecies name to each of the biotypes and *A. rubi*. In support of this approach, they noted that no morphological, physiological or genotypical differentiation is possible between biotype 1 strains of *A. tumefaciens* and *A. radiobacter*, or biotype 2 strains of *A. tumefaciens*, *A. radiobacter* and *A. rhizogenes*. Their proposals avoid the problem of the correlation between phytopathogenicity and the presence of Ti-plasmids. Under other classifications, strains harboring this plasmid and causing tumours have been classified as *A. tumefaciens*, and those without such plasmids and not causing tumours as *A. radiobacter*. However, Ti-plasmids can be lost, transferred and gained under natural conditions so that strains assigned to species on the basis of pathogenicity can change their species status according to the presence or absence of the plasmid. A stable taxonomy for *Agrobacterium* cannot be built on such a basis.

A logical taxonomy based on the above principle would be (Kersters and De Ley, 1984):
(a) *A. radiobacter* (type species) with *A. radiobacter* pv. *tumefaciens* for tumorigenic biotype 1 strains.
(b) A new *Agrobacterium* species (still to be named) for the majority of the biotype 2 strains.
(c) *A. rubi* for the separate *A. rubi* cluster.
The status and name of the biotype 3 strains should be decided later when more information is available.

Although the abovementioned nomenclature seems a good solution in which the species are differentiated by genotypic and phenotypic criteria, it could not be implemented as it was

blocked by Opinion 33 of the Judicial Commission (1970), designating *A. tumefaciens* as the type species. In Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) the genus *Agrobacterium* is therefore divided into species as follows (Kersters and De Ley, 1984):

(a) *A. tumefaciens*, comprising tumorigenic strains belonging to biotypes 1, 2 and 3.

(b) *A. radiobacter*, comprising non-tumorigenic strains belonging to biotypes 1 and 2.

(c) *A. rhizogenes*, comprising rhizogenic strains belonging to biotypes 1 and 2.

(d) *A. rubi*.

This classification system provides the freedom to add other biotypes to the species when necessary. However, three out of the four species do not reflect the true biological and taxonomic subdivisions of the genus *Agrobacterium*, as the real taxonomic entities are represented by the biotypes. Until recently no non-pathogenic *A. radiobacter* biotype 3 had been isolated from nature. However, non-pathogenic *A. radiobacter* biotype 3 isolates have now been obtained in South Africa by Staphorst *et al.* (1985).

Whatever the final decisions on nomenclature and taxonomy, it is clear that there are three separate biotypes of *A. tumefaciens* (as at present defined) that induce crown gall (Keane *et al.*, 1970; Kerr, 1974; Kerr and Panagopoulos, 1977; Süle, 1978). It is also clear from many studies of the biochemical and physiological properties of *Agrobacterium* strains (Butler and Jones, 1949; Keane *et al.*, 1970; Kerr and Panagopoulos, 1977; Kiraly *et al.*, 1970; Panagopoulos and Psallidas, 1973; Spiers, 1979; Süle, 1978) that the same biotypes (except biotype 3) occur in the species *A. tumefaciens*, *A. radiobacter* and *A. rhizogenes*. As these species names indicate pathogenicity determined by the Ti-plasmid (Kerr, 1978; Montoya *et al.*, 1977) while the biotype indicates biochemical and physiological properties, it is obvious that biochemical or physiological properties cannot be used to distinguish pathogenic (except perhaps *A. rubi*) from non-pathogenic *Agrobacterium* strains.

Recognition and Significance of *Agrobacterium* Biotypes

More than two decades before the two main biotypes of *A. tumefaciens* were recognised and given biotype designations, Wormald (1945) reported that the species contained three or four types that were morphologically similar, but differed in their pathogenicity, nutrition and biochemical properties. Later, the *A. tumefaciens* strain of Kerr (1969a), because of differences from *A. tumefaciens* as described in Bergey's Manual (Breed *et al.*, 1957), was designated biotype 2, and the typical strain biotype 1 (Keane *et al.*, 1970). *Agrobacterium* isolates can be assigned to those two biotypes on the basis of biochemical tests (Kerr, 1974; Kerr and Panagopoulos, 1977). These tests included the production of 3-ketolactose, growth in a 2% sodium chloride solution, maximum growth temperature, reaction in litmus milk, acid production from erythritol and melezitose, alkali production from malonate, propionate and tartrate and growth on different selective media. Most characters of *A. tumefaciens* are stable enough for distinguishing the biotypes (Panagopoulos and Psallidas, 1973) and the differences are even sufficient, according to Moore (1979) and Kersters and De Ley (1984), for the elevation of the two biotypes to species (see previous section).

Biotypes 1 and 2 of Keane *et al.* (1970) correlate with groups I and III of White (1972) and clusters 1 and 2 of Kersters *et al.* (1973). However, the biotype designation became generally accepted, for example, by Kerr and Panagopoulos (1977), Lopez (1978), Miller and Vruggink (1981), Panagopoulos and Psallidas (1973), Spiers (1979) and Süle (1978). Spiers (1979) also isolated an intermediate group, which differed from all three of the known biotypes, but which he designated biotype 1-2. Genetic mutations and recombinations between *Agrobacterium* strains do occur in nature, and it is to be expected that intermediate groups between biotypes will develop. However, intermediate biotypes are scarce in comparison with agrobacteria of the large biotype 1 and 2 groups (Moore *et al.*, 1980). When additional cell characteristics were investigated by Du Plessis *et al.* (1984), they found that their main clusters of strains corresponded with the biotypes but small clusters or groups could be recognised within the biotypes.

Most strains within biotype 1 or 2 were found by Kerr (1969a) to be similar in the biotype tests, even though they were obtained from different hosts and different localities. All *Agrobacterium* strains from stone fruits belong to biotype 1 or 2 (Panagopoulos *et al.*, 1979). Biotype 1 and 2 strains can occur together in soils (Kerr, 1969a) and even in the same tumours (Alconero, 1980; Panagopoulos and Psallidas, 1973). In certain countries biotype 2 strains seem to be more prevalent than biotype 1 strains (López, 1978; New and Kerr, 1971; Panagopoulos and Psallidas, 1973; Süle, 1978). However, Süle (1978) could find no correlations between biotype and geographical location.

A third biotype, biotype 3, has been isolated from grapevines (Kerr and Panagopoulos, 1977; Panagopoulos and Psallidas, 1973) as well as from *Chrysanthemum* species (Bazzi and Rosciglione, 1982). However, in South Africa (Loubser, 1978; Staphorst *et al.*, 1985; Van Zyl *et al.*, 1986) and Hungary (Süle, 1978), biotype 3 has so far been isolated only from grapevines. The majority of isolates from grapevines have been biotype 3 (Panagopoulos *et al.*, 1979). Biotype 3 strains appear not to be pathogenic on the other horticulturally important crown gall hosts including pear, apple, tomato, rose and almond (Panagopoulos and Psallidas, 1973), thus they seem to have a much smaller host range (predominantly the grapevine) than biotype 1 and 2 strains (Panagopoulos *et al.*, 1978). This specificity of biotype 3 strains is of great epidemiological significance, as fruit trees and grapevines are often planted close together. Biotype 3 strains are sometimes found together with the other two biotypes in soils and tumours (Panagopoulos and Psallidas, 1973; Süle, 1978).

As indicated in the previous section on taxonomy, biotype 1 and 2 strains consist of both pathogens and non-pathogens (Keane *et al.*, 1970; Panagopoulos and Psallidas, 1973), hence the biotyping of isolates cannot distinguish between pathogens and non-pathogens (Abo-El-Dahab *et al.*, 1978; Moore *et al.*, 1980; Schroth *et al.*, 1971) and thus cannot replace pathogenicity tests (Abo-El-Dahab *et al.*, 1978).

Procedures for the Isolation of Agrobacteria from Infected Plant Material and Soils

Isolation of *A. tumefaciens* from tumours poses many problems, and to isolate the pathogen from old and woody tumours is virtually impossible (Kiraly *et al.*, 1970). Isolations from tumours all basically follow the same procedure (Conn, 1942; Kerr, 1969b; Kerr and Panagopoulos, 1977; Lehoczky, 1968; Moore *et al.*, 1980; New and Kerr, 1972). Usually it entails the surface sterilization and removal of a part or parts of a fresh young tumour, macerating the tissue, diluting and streaking it on Petri dishes containing suitable agar media (New and Kerr, 1971). If tumour tissue on infected plant material appears to be old and therefore a poor source of *A. tumefaciens*, removal of the best parts of the tumour and the subsequent grafting of such material onto suitable indicator plants seem to be the best isolation method for the causative bacterium (Panagopoulos and Psallidas, 1973).

With a few minor modifications, the procedures for the isolation of pathogenic agrobacteria from soil are all basically the same. Soil dilutions are inoculated onto plates of suitable agar media (Kerr, 1969a; Schroth *et al.*, 1971) and after an appropriate period of incubation, typical colonies are cultured and purified. It is very often difficult to distinguish *Agrobacterium* colonies from those of other soil organisms during the isolation and subsequent purification of the bacteria (Chilton *et al.*, 1976; Kerr, 1969a; Schroth *et al.*, 1965). Pathogenicity tests (Kerr, 1969a; Miller and Vruggink, 1981; Moore *et al.*, 1980; New and Kerr, 1971; Panagopoulos *et al.*, 1979; Schroth *et al.*, 1971) are performed according to Koch's postulates. The inoculated plants are grown in moist chambers for a period adequate for symptom expression (Spiers, 1979).

Schroth *et al.* (1965) developed a selective medium for the isolation of biotype 1 *Agrobacterium* strains and New and Kerr (1971) a selective medium for the isolation of biotype 2 *Agrobacterium* strains from soils. The value of these two media for selection of the respective biotypes was confirmed subsequently by Miller and Vruggink (1981), Moore *et al.* (1980), Panagopoulos *et al.* (1979) and Schroth *et al.* (1971), although certain strains of biotypes 1 (Sonoki *et al.*, 1978) and 3 (Merlo and Nester, 1977) grew on the medium of New and Kerr

(1971). Other selective media for biotype 1 strains were proposed by Clark (1969) and Kado and Heskett (1970), but these yielded little growth (Clark, 1969; Merlo and Nester, 1977) and certain biotype 2 strains grew on the medium of Kado and Heskett (1970). Kerr (1969a) found the medium of Schroth *et al.* (1965) unsuitable for the isolation of biotype 1 pathogens. No selective medium was available for the biotype 3 pathogens (Kerr and Roberts, 1976; Moore, 1979) until recently, when Brisbane and Kerr (1983) proposed a selective medium for each of the three biotypes.

Isolations of each biotype were achieved by Loubser (1978) using nutrient agar media after a general lack of success with the media of Schroth *et al.* (1965) and New and Kerr (1971). According to Moore (1979), these selective media are effective for isolations of agrobacteria from soils only with soil dilutions of about 1×10^{-3} g/ml. Higher soil concentrations lessen the effectiveness of the media, probably because of increasing numbers of bacteria on the plates (Moore *et al.*, 1980). Lower soil concentrations may be ineffective because of too few agrobacteria or possibly because colonies develop on the selective media only when cell aggregates are present and not from single cells (Schroth *et al.*, 1971). None of the selective media distinguishes between pathogenic and non-pathogenic agrobacteria, hence time-consuming studies of pathogenicity on suitable indicator plants are still necessary (Schroth *et al.*, 1965).

Widely used indicator plants for *A. tumefaciens* are *Datura stramonium* (jimson weed) (Anderson and Moore, 1979; Moore *et al.*, 1980; Panagopoulos and Psallidas, 1973; Schroth *et al.*, 1965), *Nicotiana glutinosa* (tobacco) and *Lycopersicon esculentum* (tomato) (Anderson and Moore, 1979; Keane *et al.*, 1970; Kerr, 1969a; Kerr and Panagopoulos, 1977; Moore *et al.*, 1980; Panagopoulos and Psallidas, 1973; Schroth *et al.*, 1965; Süle, 1978), *Helianthus annuus* (sunflower) (Anderson and Moore, 1979; Lehoczky, 1968; Moore *et al.*, 1970, 1980; Süle, 1978) and *Vicia faba* (broadbean) (Spiers, 1979). Carrot (*Daucus carota*) slices are also suitable, but are often contaminated by other soft rotting bacteria (Schroth *et al.*, 1971). On suitable herbaceous and fast growing indicator plants, tumours usually appear within 7-10 days after inoculation with the pathogen (Schroth *et al.*, 1971). Kiraly *et al.* (1970) suggested that as many

different indicator plants as possible should be used in inoculation tests, as different *A. tumefaciens* strains often differ in their host range. For example, biotype 3 strains produced tumours on sunflower (Panagopoulos and Psallidas, 1973; Süle, 1978) and tomato in the study of Süle (1978); however, they did not produce tumours on tomato, datura or almond in the study of Panagopoulos and Psallidas (1973).

Serology of *Agrobacterium tumefaciens*

In comparison with other standard bacteriological techniques, serological identifications of bacteria are much more reliable, and less time-consuming (Vruggink and Geesteranus, 1975), but depend on the availability of suitable antisera. In the case of *Agrobacterium*, various procedures have been used for immunization, antigen preparation and the demonstration of antigen-antibody reactions (Cambra and López, 1978; Keane *et al.*, 1970; Roberts and Kerr, 1974; Schilperoort *et al.*, 1969; Schroth *et al.*, 1971).

In an early study, Riker *et al.* (1930) showed that groups of *A. tumefaciens, A. rhizogenes* and *A. radiobacter* were agglutinated by their strain-specific homologous antisera, but not by heterologous antisera. Berquist and Elrod (1948) found that *A. radiobacter* and *A. rhizogenes* did not react with *A. tumefaciens* antiserum, and *A. tumefaciens* did not react with *A. radiobacter* and *A. rhizogenes* antiserum. Ouchterlony plates of Schilperoort *et al.* (1969) showed differences in the precipitin lines between *A. tumefaciens* and *A. radiobacter*. However, Schroth *et al.* (1971) observed no serological differences between pathogenic *A. tumefaciens* and non-pathogenic *A. radiobacter* strains in Ouchterlony double diffusion tests.

Roberts and Kerr (1974) compared the antigens of a number of non-pathogenic *A. radiobacter* strains before and after their conversion to pathogenic strains. No serological differences could be found, in Ouchterlony double diffusion experiments, between the pathogens and non-pathogens. Hochster and Cole (1967) and Graham (1971) could detect no serological differences between virulent and avirulent *Agrobacterium* strains. Keane *et al.* (1970) discovered with tube agglutination and direct immunofluorescence, and López (1978) with

indirect immunofluorescence, serological differences between *A. tumefaciens* biotype 1 and 2 strains. Only a few biotype 3 strains, with the exception of the homologous antigen, reacted with the biotype 3 antiserum in immunofluorescent and immunodiffusion tests (Miller and Vruggink, 1981). These serological results using *A. radiobacter* subsp. *tumefaciens (A. tumefaciens*), showed the existence of several serotypes, which could be important in epidemiological studies.

The genus *Agrobacterium*, especially *A. tumefaciens*, seemed to be a heterogeneous group of organisms on the basis of somatic antigen reactions in agglutination tests. Among Gram-negative bacteria, such as the Enterobacteriaceae, Kauffman (1966) found that the LPS O-antigen portion of the cell walls provided a wide range of antigens which were most useful for primary serological grouping. Graham (1971), in a study of LPS cell wall fractions by immunodiffusion and immuno-inhibition reactions, discovered five different LPS groups among nine strains of *Agrobacterium*. However, species could not be distinguished on the basis of their serological reactions.

As shown in studies with other Gram-negative plant pathogenic bacteria and *Rhizobium*, various factors influence serological tests with extracted or fractionated cells, such as those used by Graham (1971) and Miller and Vruggink (1981). Different methods of extraction or disintegration can be used to expose additional diffusible antigens (Humphrey and Vincent, 1965). Generally heat treatments have been used, as they apparently do not destroy the somatic (O) antigens; however, they caused dissociation of antigens into non-antigenic subunits in *Erwinia carotovora* (De Boer *et al.*, 1979). Phenol treatment has also been used and seems to be an easier, quicker and better method to extract diffusible antigens than the conventional heat treatment (Vruggink and Geesteranus, 1975). The extent to which the structure of the bacterial cell wall is disrupted by the antigen-releasing treatment is important in determining which antigens become available as diffusible antigens for precipitation by antisera. The fact that very strong reagents are usually used to extract specific antigenic determinants of bacterial cell walls, suggests that cell walls may retain antigens in a non-diffusible state during general antigen-releasing treatments. For example, the somatic O-antigens as structural parts of the

cell wall may possibly react in this way and be available only for agglutination and not diffusion (Humphrey and Vincent, 1965). Differences in precipitation lines can occur in immunodiffusion tests depending on whether the immunizing antigens are whole or disintegrated cells (Graham, 1971; Humphrey and Vincent, 1965; Miller and Vruggink, 1981). Humphrey and Vincent (1965) also found that *Rhizobium trifolii* released additional antigens when grown on a calcium-deficient medium, in comparison to organisms grown on a medium containing sufficient amounts of calcium. They attributed this result to the autolysis of the more fragile calcium-deficient bacteria.

Cambra and López (1978) successfully applied the indirect ELISA method to *A. tumefaciens* identification, and found it to be eight times more sensitive than the indirect immunofluorescence technique. The ELISA method seems to have potential for *A. tumefaciens* detection in soils and detection of common and different antigens in different *Agrobacterium* strains. Small differences could possibly be detected by the indirect ELISA method using either soluble or whole cell antigens. Kishinevsky and Bar-Joseph (1978), Kishinevsky and Gurfel (1980) and Morley and Jones (1980) used the ELISA method in studies of *Rhizobium* strains. Kishinevsky and Bar-Joseph (1978) demonstrated that the *Rhizobium* serogroups defined by the ELISA test were similar to those defined by agglutination and immunodiffusion tests. The sensitivity of the ELISA method was four to six orders of magnitude higher than immunodiffusion and agglutination, so that specific *Rhizobium* strains could be detected at concentrations of 10^4 to 10^5 cells/ml. Agglutination and immunodiffusion tests detected these bacteria only at concentrations of 10^9 to 10^{10} cells/ml. The ELISA technique could be used for *Rhizobium* strain identification in mixed cultures. Morley and Jones (1980) also detected *Rhizobium* strains in root nodules; in their investigations, instead of using the chromogenic substrate p-nitrophenylphosphate, they used a fluorogenic substrate (3-0-methylfluorescein phosphate) to increase sensitivity and permit the use of a low titre antiserum.

The specificity of serological reactions has led to the development of practical serological methods for accurate identification of bacterial plant pathogens (Trigalet *et al.*, 1978).

Agglutination and precipitation methods were initially used for the identification of certain phytopathogenic bacteria which could otherwise not be distinguished readily from other bacteria (Hubalek, 1982; Trigalet *et al.*, 1978). In more recent times the need has been for faster and more effective serological identification procedures. The ELISA technique saves much time and numerous samples can be screened simultaneously on a very economical basis (Afanador and Victoria, 1981). In respect of diversity, sensitivity, precision and speed with which results are obtained, the ELISA technique is far superior to other serodiagnostic methods (Clark, 1981). An important feature of immunosorbent assays is their ability to detect plant pathogenic bacteria at much lower concentrations than the classical immunoprecipitin methods (Clark, 1981). The ELISA method has a high quantitative potential, due to the fact that the indicator-liberating enzyme attached to the antiserum becomes a function of the bacterial concentration in the wells (Vruggink, 1978). The method can detect antigens of different sizes and morphology (Clark, 1981) and could be used for latent infection and epidemiological studies (Nomé *et al.*, 1980). The only disadvantage of the ELISA system is the high threshold value (10^4-10^6 cells/ml) where bacteria can be detected. Below this value, the bacteria, although present, cannot be identified.

In the recent papers on *Agrobacterium* serology by Digat (1978) and Miller and Vruggink (1981), differing opinions have been expressed on the possibility of using serodiagnosis for the identification of bacterial pathogens. According to Digat (1978), antigenic specificity of plant pathogenic bacteria is closely related to the bacterial genus and species, with specificity being often more prevalent for pathogenic species with a narrow host range. However, Miller and Vruggink (1981) claimed that routine serological diagnosis seems impossible, as there is relatively little serological resemblance between similar biotypes, pathogenic or non-pathogenic strains.

Whether serological diagnosis is successful or not will obviously depend on the antigenic structure of the agrobacteria under investigation. Like other Gram-negative bacteria, the antigenic structure of the agrobacteria can be regarded as a mosaic of antigenic determinants. Some may be specific for a particular strain or nomen species, while others may be distributed

between more or less related species, or even species not sufficiently related to permit their inclusion in the same bacterial genus, e.g. in the case of heterophylic antigens. The ideal approach for diagnosis, therefore, as outlined by Trigalet *et al.* (1978), would be to determine the antigenic determinant responsible for the defined specificity, and to produce a monospecific antiserum against this antigen.

Biological Control of Crown Gall

Stonier (1960) discovered two *Agrobacterium* strains which produced a bacteriocin which he called agrobacteriocin I. This bacteriocin inhibited 12 other *A. tumefaciens* strains and one *A. radiobacter* strain *in vitro*.

New and Kerr (1972) were the first to report biological control of crown gall by the bacteriocinogenic strain *A. radiobacter* strain 84 (or K84). Kerr (1974) obtained excellent control of the disease on potted peaches planted in containers filled with natural field soil. The most dramatic effect was achieved using soil naturally infested with both biotypes 1 and 2 of *A. tumefaciens*. Three inoculation treatments with strain K84 were applied:

(a) seed inoculation only,

(b) root inoculation only, and

(c) both seed and root inoculation.

Approximately 95% control was achieved by root treatment, and 99% control through combined seed and root treatment. This control was attributed to the action of a bacteriocin that inhibited the growth of *A. tumefaciens in vitro* (Kerr, 1974; Moore, 1979). *Agrobacterium radiobacter* strain 84 was insensitive to its own bacteriocin. A high correlation existed between *in vitro* agrocin sensitivity of *A. tumefaciens* strains and biological disease control in a tomato plant experiment, although this may not have been a true reflection of control in the field (Kerr, 1974). New and Kerr (1972) and Kerr (1974) obtained complete inhibition in their *in vivo* biological control tests with a 1:1 ratio of pathogen to control organism applied to the test plants.

Following the early pioneering work in Kerr's laboratory (New and Kerr, 1972; Kerr, 1974), biological control with *A. radiobacter* K84 was rapidly adopted so that it has become the most widely applied biological control of any plant disease (Moore and Warren, 1979). Kerr and Panagopoulos (1977) noted that the successful biological control examples were truly impressive. Moore (1979) stated that crown gall prevention by *A. radiobacter* K84 was one of the most successful examples of biological control in plant pathology, and the first case of biological control of a bacterial disease.

Kerr (1980) reported that *A. radiobacter* K84 had, since 1973, been used successfully in Australia in stonefruit and rose nurseries. The high control percentages achieved in Australia (Kerr, 1974) and New Zealand (Moore and Warren, 1979) were attributed to most pathogens belonging to biotype 2, having very little natural resistance to the *A. radiobacter* K84 control. Kerr (1974) attributed the successful control of crown gall in Australia to the very homogeneous population of bacteriocin-sensitive *A. tumefaciens* strains.

López (1978) found that 20 virulent *A. tumefaciens* strains in France, consisting mainly of biotype 2 organisms, were sensitive to the agrocin of *A. radiobacter* K84. Spiers (1980a) reported a 98% *in vitro* antagonism of pathogenic *A. tumefaciens* biotype 1 strains by *A. radiobacter* K84 and in a separate study, not involving the same *A. tumefaciens* pathogens, complete control of crown gall on potted *Salix* species (Spiers, 1980b). Bazzi and Mazzucchi (1978) obtained greater than 90% control of *A. tumefaciens* in Italy on peaches and cherries. López *et al.* (1981) used the control strain in Spain and achieved 74% control of the pathogen on rose plants. Matthee *et al.* (1977) applied *A. radiobacter* K84 in South Africa to about 6 000 peach seedlings in soils that were highly contaminated by the pathogen giving greater than 10% infection without control. A control of 98% was achieved by seed treatments alone. Schroth and Moller (1976) achieved almost 100% control of crown gall on stone fruit trees in California, even when the pathogen was applied before *A. radiobacter* K84. Dhanvantari (1976) achieved successful control of the pathogen on peach seedlings in Canada. Grimm and

Süle (1981) found *A. radiobacter* K84 much more effective on peaches and cherries than on apple rootstocks.

Hendson *et al.* (1983) reported the isolation of *A. tumefaciens* strain D286 from a *Eucalyptus* sp. in South Africa. This isolate was found to produce an agrocin working in a similar fashion to the agrocin 84 produced by *A. radiobacter* K84. At a later stage *A. tumefaciens* D286 also spontaneously lost its pathogenicity. Hendson *et al.* (1983) found agrocin D286 to have a broader host range than agrocin 84. Agrocin D286 was also found to be active against *A. tumefaciens* strains harbouring nopaline, octopine, or agropine Ti-plasmids (Hendson *et al.*, 1983), whereas agrocin 84 was only active against *A. tumefaciens* strains harbouring nopaline Ti-plasmids (Engler *et al.*, 1975). In other words, agrocin D286 inhibited some *A. tumefaciens* strains that were resistant to agrocin 84. It was also found that more agrocin D286 than agrocin 84 was produced by the bacteria in culture. This was probably due to the faster growth rate of *A. tumefaciens* D286 compared to that of *A. radiobacter* K84. Kerr and Tate (1984) discovered that neither agrocin 84 nor agrocin D286 inhibited *A. tumefaciens* biotype 3 strains.

In evaluating the potential of a biological control agent, knowledge of the biology of the pathogen and the disease it causes, its geographic distribution, and the edaphic factors that contribute to disease expression is essential (Linderman *et al.*, 1983). An organism successful as an antagonist in tests *in vitro* often fails in greenhouse and field trials. Critical information is, for example, knowledge of the organic matter content, pH, nutrient and moisture levels of the soil from which the potential biocontrol agent was isolated. As the organism had that particular soil as a habitat it may possibly function only in soil conditions nearly matching the original conditions. Certain soil variables may nullify or modify biocontrol, for example, alteration of the soil pH by only one unit may drastically affect the efficacy of a biocontrol test organism (Scher and Baker, 1980). The introduction of soil antagonists into the rhizosphere is difficult as the rhizosphere is usually biologically buffered or fully occupied. The antagonist's greatest biocontrol potential is in preventative treatments and it should therefore normally be applied before the pathogen is introduced (Linderman *et al.*, 1983).

According to Ellis *et al.* (1979), a successful biological control organism for crown gall should have two major characteristics, namely;

(a) it must produce an effective bacteriocin in sufficient quantities, and

(b) it must grow well and produce enough bacteriocin to inhibit the pathogen on the surface of the potential host.

Ellis *et al.* (1979) assumed that the basic mechanism of biological control by *A. radiobacter* strain K84 was inhibition of the pathogen by agrocin 84.

Roberts *et al.* (1977) identified agrocin 84 as a 6-N-phosphoramidate, a 'fraudulent' adenine nucleotide. Kerr and Tate (1984) described the agrocin as an $N^6$5'-disubstituted adenine nucleotide with a fraudulent nucleoside sugar. The alpha face of the β-D-3'deoxyarabinofuranosyl group is structurally equivalent to a dideoxynucleoside, which is an ideal analogue for DNA chain termination.

Agrocin D286 produced by the South African strain *A. tumefaciens* D286 was found to resemble agrocin 84, having the characteristic adenine-N^6-phosphoramidate UV absorption maximum at 264 nm. However, the wider specificity range makes the nature of the substituents a matter of considerable interest (Hendson *et al.*, 1983).

Agrobacterium radiobacter K84 contains a large plasmid coding for nopaline catabolism and conjugation, and a smaller plasmid coding for the production of agrocin 84 and resistance to this agrocin (Ellis and Kerr, 1978, 1979; Ellis *et al.*, 1979; Merlo and Nester, 1977). The genes controlling the sensitivity of pathogenic agrobacteria to agrocin 84 are located on the Ti-plasmid (Engler *et al.*, 1975; Kerr, 1975; Schell, 1978). There seems to be a very close linkage on this plasmid between the genes for pathogenicity and sensitivity to agrocin 84 (Kerr, 1980). However, not all tumour-inducing Ti-plasmid-carrying *A. tumefaciens* strains are sensitive to agrocin 84 (Engler et al., 1975). It was proposed by Engler *et al.* (1975) that the Ti-plasmid of resistant strains does not carry the genes for sensitivity.

Moore (1979) and Cooksey and Moore (1982a) tested the hypothesis that *A. radiobacter* K84 inhibited *A. tumefaciens* infections by liberating an extracellular bacteriocin which inactivated sensitive *A. tumefaciens* strains. Dead *A. radiobacter* K84 cells (Moore, 1979), and a non-bacteriocin-producing mutant of *A. radiobacter* K84 (Cooksey and Moore, 1982a), did not inhibit tumour formation when inoculated onto plants together with a K84-sensitive *A. tumefaciens* strain, but did inhibit tumour formation if they were inoculated onto the plants 24 h prior to the pathogen. Cooksey and Moore (1982a) discovered that a cell-free filtrate of an *A. radiobacter* K84 culture inhibited tumour formation by the pathogen. However, Moore (1979) noted that agrocin 84 had never been isolated from tumour wound tissues and Cooksey and Moore (1982a) proposed that control might be the result of both agrocin 84 action and a physiological blockage of infection sites by *A. radiobacter* K84. Following a scanning electron microscope study, Smith and Hindley (1978) proposed that agrocin 84 blocked the initial step of tumour induction, namely, the attachment of the pathogen to the host cell wall. It seemed that the agrocin acted by upsetting the integrity of the cell wall of the pathogen and fragmentation or elimination of the bacterial cell wall was proposed.

According to Reeves (1972), bacteriocins attach to receptor sites on the cell walls of bacteria against which they act. Roberts and Kerr (1974) found agrocin 84 to react in this way. Kerr and Htay (1974) and Roberts and Kerr (1974) hypothesized that virulent bacteria must have a specific molecular configuration on their surfaces which are involved in pathogenesis, and when this configuration changes their pathogenicity is lost as they cannot attach to host cell walls. Roberts and Kerr (1974) proposed that this configuration might also be the receptor site for agrocin 84 attachment.

Lippincott and Lippincott (1980) found that the numbers of tumours caused by a virulent pathogen decreased when avirulent strains were added to the inoculum, but not all avirulent cells had the same effect. They proposed attachment of the avirulent cells to a specific host site, thereby excluding the pathogen. Heat-inactivated pathogenic cells had the same inhibitory effect.

Genes for agrocin sensitivity are located on the nopaline Ti-plasmid (Engler et al., 1975). They code for agrocin 84 uptake by a high affinity transport system in which the agrocin is bound to a protein fraction in the periplasmic space of a sensitive strain (Murphy and Roberts, 1979). Ellis and Murphy (1981) found four new opines while searching for a non-toxic substrate for agrocin 84 permease, which were all phosphodiesters which interacted with agrocin 84. Agrocinopines A and B were found in tumours induced by nopaline strains, and agrocinopines C and D in those tumours induced by agropine strains. Agrocinopines A and B in nopaline tumours induce the transfer of nopaline Ti-plasmids. Agrocinopines C and D are found in agropine tumours and induce the transfer of agropine Ti-plasmids. These opines are phosphorylated sugar derivatives.

According to Kerr and Tate (1984), *A. radiobacter* strain K84 appears to be a 'pirate' of the crown gall system. It has genes for the catabolism of nopaline and can therefore compete with the real pathogens for this substrate. Pathogenic *A. tumefaciens* strains with genes for nopaline utilization have two genes for the synthesis of two distinct kinds of opine, for example, nopaline and the related nopalinic acid on the one hand and agrocinopine A and B on the other. In addition to being competitive, *A. tumefaciens* K84 may kill these pathogens by producing agrocin 84 which is actively transported into the pathogens via a permease designed for the uptake of agrocinopine(s).

During a search for alternatives to *A. radiobacter* K84 for crown gall control Alconero (1980) found that none of 173 other non-pathogenic *Agrobacterium* strains appeared to be antagonistic towards 21 A. tumefaciens strains of both biotype 1 and 2. However, Cooksey and Moore (1980) showed that bacteria and fungi of certain genera (*Bacillus*, *Pseudomonas*, *Aspergillus*, *Penicillium*) excreted strong antibiotic substances which reduced tumour counts on cherry seedlings.

Resistance of *Agrobacterium tumefaciens* to Agrocin 84 and Breakdown of Biological Control

New and Kerr (1972) found most (6/8) *A. tumefaciens* biotype 2 strains tested to be sensitive to *A. radiobacter* K84, but postulated that an organism not controlled by *A. radiobacter* K84 would have a biological advantage over agrocin-sensitive strains, and would therefore be selected when control was applied. Kerr and Htay (1974) found only one out of 45 *A. tumefaciens* strains to be resistant to *A. radiobacter* K84. They stated that the biggest problem will be to control the *A. tumefaciens* strains that are insensitive to agrocin 84. They reported that resistance did not appear to be a problem in Australia because 29/30 pathogens were sensitive to agrocin 84; however, 7/10 overseas *A. tumefaciens* strains were resistant to agrocin 84 and not subject to biological control. In parts of Europe (Kerr and Htay, 1974), United States of America (USA) (Schroth and Moller, 1976) and Greece (Kerr and Panagopoulos, 1977), there were some cases where no evidence of control was found. Moore (1979) also reported that pathogens resistant to *A. radiobacter* K84 occurred naturally in the USA. *Agrobacterium radiobacter* K84 gave no control against latent infections (Moore, 1979; Moore and Warren, 1979). Spiers (1980b) found that *A. radiobacter* K84 caused little control of tumour formation on *Salix* species in the field because of resistant pathogenic strains of *A. tumefaciens*. No biological control was found by Van der Scheer (1980) on raspberry in the Netherlands. Grimm and Süle (1981) also discovered that the number of tumours was two to three times higher on the M9 apple rootstock when *A. radiobacter* K84 was applied compared to the treatment where it was not applied. High proportions of *A. tumefaciens* isolates resistant to *A. radiobacter* K84 were reported by Alconero (1980) in South Carolina (36%) and Tennessee (24%), USA. He also reported that 7% of all tumours investigated contained both biotype 1 and 2 strains, but he did not state whether these strains were sensitive to agrocin 84 or subject to biological control. He found that sensitive pathogens varied in their sensitivity towards *A. radiobacter* K84, so that some of the weakly sensitive strains needed a 3:1 or 10:1 ratio of *A. radiobacter* K84:pathogen to achieve control.

Kerr (1975) discovered a close correlation between the ability of *A. tumefaciens* strains to utilize octopine and their resistance to agrocin 84. Agrocin-resistant strains utilized octopine,

whereas sensitive strains did not. Kerr and Panagopoulos (1977) found only 41 *A. tumefaciens* strains out of 53 to be controlled by *A. radiobacter* K84 and subject to biological control in glasshouse tests. Twenty-seven of the controllable pathogenic strains belonged to the biotype 2 and 14 to the biotype 1 group. The 12 resistant pathogenic strains comprised nine biotype 3 isolates from grapevine and three biotype 2 isolates from peach. None of the biotype 3 pathogens was sensitive to agrocin 84 or subject to biological control. Ellis and Kerr (1978) reported nopaline-catabolizing biotype 2 strains to be the biggest pathogen group in Australia. All such strains were biologically controllable. Octopine-catabolizing biotype 1 strains were insensitive to agrocin 84, and not biologically controllable. Bazzi and Mazzucchi (1978) found in Italy that tumorigenic non-3-ketolactose-producing strains (biotype 2) were more sensitive to agrocin 84 than the 3-ketolactose producing (biotype 1) strains. Scalza *et al.* (1979) in the USA sprayed an *A. radiobacter* K84 suspension onto peaches and peach seeds in open furrows in areas where the soil was infested naturally with the *A. tumefaciens* pathogen. In test plots which were fumigated prior to inoculation, there appeared to be some breakdown in biological control.

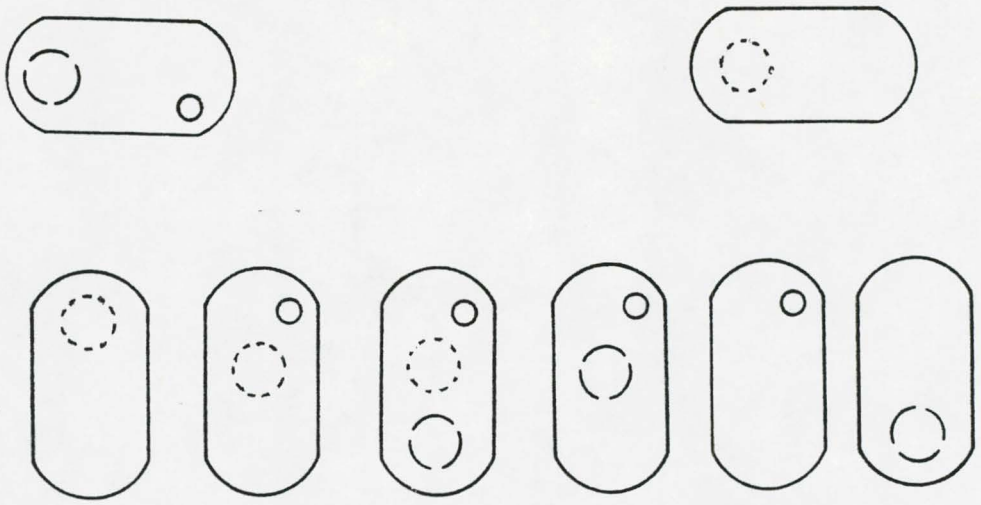
Panagopoulos *et al.* (1979) confirmed a serious breakdown in biological control in Greece in field and glasshouse experiments and studied strains from tumours to investigate the mechanism of breakdown of the biological control. When the pathogen and *A. radiobacter* K84 were applied together in a 1:1 ratio to plants, many tumours developed from which pathogenic, agrocin-producing strains were subsequently isolated. These strains were resistant to agrocin 84 and thus not controllable. Ellis and Kerr (1979) and Ellis *et al.* (1979) found that the agrocin 84 plasmid could be transferred to non-pathogenic *Agrobacterium* strains. The agrocin 84 plasmid was not conjugative, but *A. radiobacter* K84 has a nopaline plasmid, which is conjugative. This plasmid promoted both conjugation and mobilization of the agrocin 84 plasmid. Panagopoulos *et al.* (1979) successfully used virulent recipients for the agrocin 84 plasmid with or without the nopaline plasmid. They believed that the partial failure of control was caused mainly by the development of bacteriocinogenic pathogens which, being resistant to agrocin 84, would have increased in number and induced the galls on treated plants.

The possible breakdown in biological control with *A. radiobacter* K84 also seemed to Ellis and Kerr (1979) and Kerr (1980) to be a result of the formation of recombinants. Ellis and Kerr (1979) actually determined through crosses between agrobacteria that such transfers could take place. In theory their crosses would produce six progeny types (Fig. 1) and all six types were isolated (Kerr, 1980). Transconjugants B and C combined pathogenicity with production of and resistance to agrocin 84. As nopaline which is required for the conjugation promoted by the nopaline plasmid, is present naturally only in crown gall tumour tissue, this type of plasmid transfer can occur under natural conditions only within the tumour tissue.

A problem in laboratory tests of agrocin 84 resistance is that different growth media can influence the susceptibility of pathogenic *Agrobacterium* strains towards the agrocin (Smith and Hindley, 1978; Moore and Warren, 1979). Furthermore, Spiers (1980a) found that the production of agrocin 84 by *A. radiobacter* was influenced by the growth medium, incubation time and temperature. Agrocin production was directly related to the growth of the producer bacteria, so that factors reducing growth had an inhibitory effect on agrocin production. The host plant species also influenced the interaction between *A. radiobacter* K84 and the pathogen (Smith and Hindley, 1978; Moore and Warren, 1979). According to Moore and Warren (1979) control of *A. tumefaciens* underground by *A. radiobacter* K84 depends on factors such as root metabolism, competitive microbes and fauna, soil salts, moisture, gases, temperature and pH, and can vary from region to region. Moore (1977) discovered examples of pathogenic strains which were resistant *in vitro* but were controlled by *A. radiobacter* K84 in the field, while Kerr and Panagopoulos (1977) found strains which produced agrocin *in vitro* but failed to control sensitive strains in the field.

Süle and Kado (1980) suggested that an impairment of the agrocin uptake mechanism could lead to resistance. Kerr and Htay (1974) and Engler *et al.* (1975) discovered that resistant biotype 1 *A. tumefaciens* strains developed in the presence of agrocin 84. These resistant colonies appeared to have lost their Ti-plasmids and were no longer pathogenic. Kerr and Htay (1974) also discovered a pathogenic strain, *A. tumefaciens* strain 108, which secreted a bacteriocin that inhibited *A. radiobacter* K84.

K84 DONOR X PATHOGEN RECIPIENT



Type	A	B	C	D	E	F
Parental	+	-	-	+	-	-
New (gain)	-	+	+	-	-	-
New (loss)	-	-	-	-	+	+
Pathogen-icity	+	+	+	-	-	-
Agrocin production and resistance	-	+	+	+	+	-
Nopaline Utilization	-	-	+	+	-	+

Fig. 1. Hypothetical result of a crossing between *A. radiobacter* K84 and a pathogenic recipient, according to Kerr (1980). Chromosomes are not shown. The one donor plasmid (small circle, ○)codes for agrocin production and resistance. The other donor plasmid (broken circle,◠ ◡) codes for nopaline utilization and conjugation. The plasmid (Ti-plasmid) of the pathogen recipient (dotted line circle,◌) codes for pathogenicity, sensitivity to agrocin 84 and several other characters.

In view of the observations that biological control of *A. tumefaciens* with *A. radiobacter* K84 does not work in all soils, López et al. (1981) suggested that extensive field trails would have to be done on inoculated and uninoculated hosts before the control procedure could be used in practice. To prevent the possibility of transfer of the agrocin 84 plasmid from *A. radiobacter* K84 to *A. tumefaciens* and the breakdown of crown gall control, a mutant of *A. radiobacter* K84 with a defective plasmid transfer system should be used (Kerr, 1980).

López *et al.* (1981) found *A. radiobacter* K84 to be sensitive to certain fungicides *in vitro*. Captan (3 g/l) completely inhibited the growth of *A. radiobacter* K84, and a slight inhibition was found with benomyl (1 g/l). The inhibitory effect of captan could not be proven in field studies, in spite of a decline in biological control of a bacteriocin-sensitive *A. tumefaciens* strain on tomato plants when captan was applied together with *A. radiobacter* K84.

Deep *et al.* (1968) reported that the incidence of crown gall increased after certain soil fumigations, possibly by elimination of some of the competitive microorganisms. This phenomenon is not necessarily related to agrocin 84 production.

Chemical and Physical Methods for Controlling Crown Gall

Melhus and Maney (1921) discovered that apple scions dipped in a Bordeaux mixture ($CuSO_4$, 50 g/l; lime, 50 g/l) and wrapped lightly with a cotton bandage showed no phytotoxic effects, while crown gall was eradicated successfully.

According to Schroth and Hildebrand (1968) and Falk (1977), Gallex (2,4-xylenol, 4.63 g/l; meta-cresol, 4.66 g/l; inert liquid ingredients, 990.71 g/l), controlled crown gall tumours *in situ* on plants, although phytotoxicity levels were relatively high. This product, previously known as Bacticin, was originally developed by Schroth and Hildebrand (1968) and later licensed by the University of California, USA. Tumours were prepared for treatment by the removal of soil with water and allowed to dry for one or more days. Most of the tumour was also removed.

Gallex was then painted on the tumour and cut surfaces as well as onto a 1-cm-wide zone of surrounding healthy bark. The Gallex had to be applied before the breaking of dormancy or during the early growing season. However, Ferreira (1985) found Gallex, as well as streptomycin sulphate (0.5 g/l), oxytetracycline (0.1 g/l), natamycin (0.1 g/l) and a 42:58 copper sulphate-lime mixture to cause no significant disappearance of crown gall tumours from grapevines. These chemicals were all applied by brush in late summer to the surface of wounds where tumours were removed. All treatments were repeated 12 months later after the removal of newly formed tumours. The antibiotics chloramphenicol and oxytetracycline were also infused by injection into the xylem tissue of infected vines during late summer (February). Two hundred ml of antibiotic solution were applied to each vine. No significant control of tumorous tissue was observed.

Mercury-containing chemicals have also been used in the treatment of crown gall tumours. For example, Gram and Weber (1953) noted that Wormald of the East Malling Research Station, England, used a mercury fungicide, Upsulun, which provided considerable control of crown gall on apple rootstocks. The apple rootstocks were dipped in a mixture of 50-105 g Upsulun and approximately 15 kg soil in 7 l of water. Stapp (1961) controlled crown gall by dipping roots of cutback fruit trees in a clay mush to which 1% Ceresan mercury mordant had been added.

Schroth and Moller (1976) dipped batches of almond, peach, plum and apricot seedlings for 5 sec into a 0.3 or 0.6 g/l solution of Dowco-242 (tetra-isopentyl ammonium bromide). The Dowco-242 gave excellent control of crown gall on apricots, but none on plums, almonds and peaches.

Richardson *et al.* (1976) reported a very successful chemical control (80-90%) of crown gall by application of a 0.1 M solution of D-glucosamine to tumours (1 mm wide) on garden beans (*Phaseolus vulgaris* cv. Bountiful). No phytotoxic effects were observed.

Popova and Cikova (1978) observed *in vitro* inhibition of pathogenic *A. tumefaciens* and control of tumour formation by an extract prepared from the weed *Hypericum perforatum* (St. John's Wort). They applied the extract to *A. tumefaciens* in Petri dishes, and also in different concentrations to crown gall tumours.

Soil solarization (using a cover of transparent polyethylene plastic sheeting) for 4 weeks during the high temperature period of summer reduced *Agrobacterium* populations in the soil by up to 98% in the study of Stapleton and De Vay (1982).

General Nursery Practices for the Control and Prevention of Crown Gall

Nursery practices are very important for the control of crown gall. Disinfection of work tables, utensils and scion wax can help to curb this problem (Kerr and Htay, 1974). Moore (1976) noted that wounds on dormant Mazzard cherry seedlings were susceptible to *A. tumefaciens* infections for a period of up to 107 days. Infected plants should be removed and destroyed immediately (Gram and Weber, 1953). Because of latent infections crown gall symptoms are sometimes observed only in the second growing season (Moore, 1976); information on plant history is therefore very important, and if not available, the plants should undergo a quarantine inspection for an adequate period of time (New and Kerr, 1971; Butler and Jones, 1949). The source of irrigation water is important, as water contaminated with *A. tumefaciens* can transport the pathogen in sufficient quantities to eventually cause high percentages of crown gall infection, especially in flood-irrigated areas (Smith and Cochran, 1944).

According to Rudolph (1981), breeding for resistance against crown gall still appears to be the most promising method of control.

MATERIALS AND METHODS

Agrobacterium tumefaciens Isolates and Strains

Agrobacterium tumefaciens cultures isolated from crown galls on plants in South Africa (own isolates) and those received from scientists in South Africa, Belgium and Australia are listed in Table 1. The biotype (where established in previous work), host plant from which the strains had originally been isolated and the country of origin are also indicated.

Isolation of *Agrobacterium tumefaciens* from Diseased Plant Material

Isolation from specimens from the field

Plant material collected by myself and inspectors of the Directorate of Plant and Liquor Control was visually assessed for crown gall symptoms. Typical crown gall tumours were rinsed and scrubbed with a nailbrush under running tap water.

The freshest parts were dissected from the tumours, dipped in a 5 g/l solution of sodium hypochlorite for 10 minutes, and rinsed separately five times (each time in 150 ml of sterile distilled water for 1 min). The tumour material was then macerated with a round-tipped glass rod in 2 ml of sterile distilled water in a sterile glass Petri-dish. After 30-60 min, 0.2 ml of the suspension was diluted in 10 ml sterile distilled water and 0.2 ml amounts of the diluted suspension spread onto Bacto nutrient agar (Difco Laboratories, Detroit, Michigan, USA) (NA) plates (Butcher *et al.*, 1980) using a sterile L-shaped glass rod. Diluted suspensions were also spread on plates of the selective media of Schroth *et al.* (1965), New and Kerr (1971) and Brisbane and Kerr (1983) for biotype 1, 2 and 3, respectively. The plates were incubated at 23-25^{o}C for 3-6 days and examined for the production of colonies typical of *A. tumefaciens*.

TABLE 1. Origin of isolates and strains of *Agrobacterium* species

Isolate/strain designation	Bio-type	Host plant	Isolated by	Country of origin
$M21_3$,$M21_4$,$M34_{11}$,$M37_6$, $M37_{10}$,$M37_{11}$,M37g,$M51_6$, M51g,$M52_3$,$M52_5$,M52g, $M57_2$,M129		*Chrysanthemum* sp.	F.G.H. van Zyl[a]	RSA[b]
1465,1671,1887,1895, 2153,2077		*Prunus* spp.	F.G.H. van Zyl	RSA
172(a),172(b),2080, 2086A		*Salix* sp.	F.G.H. van Zyl	RSA
78,1477,2158,2160, 2164,2221		*Vitis* sp.	F.G.H. van Zyl	RSA
D286		*Eucalyptus* sp.	T. Collett[c]	RSA
K14,K17,K21,K28,K30, 45d	1	*Chrysanthemum* sp.	H.J. du Plessis[d]	RSA
57	1	*Prunus* sp.	H.J. du Plessis	RSA
ATCC[e] 4452[f]	1	*Rubus* sp.	Unknown	USA[g]
CIP[h] B6[f]	1	*Pyrus* sp.	Unknown	USA
NCPPB[i] 925[f]	1	*Dahlia* sp.	Unknown	RSA
T-37	1	*Juglans* sp.	Unknown	USA
ICPB[j] TT9[kf]	1	*Humulus* sp.	Unknown	USA
ATCC 143[f]	1	Unknown	Unknown	USA
198	1	Unknown	A. Kerr[l]	Aus[m]
A1,A5,A6,C5,D3,D6,D7, D8,D10,39g,39i,39m,39n, 41B,46,47,48,49,70,71, 73,Z8,Z12,Z13,	2	*Prunus* spp.	H.J. du Plessis	RSA
I27	2	*Prunus* sp.	A. Kerr	Aus
Z36	2	*Vitis* sp.	H.J. du Plessis	RSA
K84	2	Soil	A. Kerr	Aus
W7,W8	3	*Vitis* sp.	H.J. du Plessis	RSA
305	3	*Vitis* sp.	A. Kerr	Aus
NCPPB 1771[f]	3	*Vitis* sp.	Unknown	Iran

[a]Plant Quarantine Station, Stellenbosch.
[b]Republic of South Africa.
[c]Previously Plant Protection Research Institute, Pretoria.
[d]Fruit and Fruit Technology Research Institute, Stellenbosch.
[e]American Type Culture Collection (Rockville, Maryland, USA).
[f]Obtained from Kersters and De Ley, Laboratorium voor Microbiologie en Microbiële Genetica (Rijksuniversiteit, Gent, Belgium).
[g]United States of America.
[h]Collection de l'Institut Pasteur (Paris, France).
[i]National Collection of Plant Pathogenic Bacteria (Harpenden, Hertfordshire, England).
[j]International Collection of Plant Pathogenic Bacteria (Davis, California, USA).
[k]Probably synonymous with B37 of Kersters *et al.*(1973).
[l]Waite Agricultural Research Institute (Adelaide, South Australia).
[m]Australia.

Isolation from indicator host plants

Tobacco (*Nicotiana glutinosa*) and datura (*Datura stramonium*) seedlings (3 weeks old) in soil in plastic pots were used for inoculations. Stems of the plants were surface-sterilized with sodium hypochlorite (5 g/l solution for 10 min), and wounded with a sterile scalpel. Wounds were approximately 0.5 cm long. Slices of rinsed tumour tissue from diseased plants were inserted into the wounds. The inoculated seedlings were maintained in a growth chamber and examined twice a week for a period of 6 weeks for the development of tumorous outgrowths. *Agrobacterium tumefaciens* was isolated from tumours on these plants in the same way as from diseased plant specimens from the field. No attempt was made to isolate *A. tumefaciens* from tumours on grapevines using this technique with tobacco and datura as host plants. Jacquez grapevine seedlings were used to isolate *A. tumefaciens* from grapevine tumours.

Isolation of *Agrobacterium tumefaciens* from Soils

Isolates of *A. tumefaciens* were obtained from soils using the dilution plate method, whereby 0.1 ml of a 1:1 000 or 1:10 000 dilution of soil in water was spread with a sterile L-shaped glass rod onto plates of NA and the selective media used for the isolation of biotypes 1, 2 and 3 from diseased plant material. The plates were incubated for up to 10 days at 23-25°C and examined periodically for the growth of colonies typical of *A. tumefaciens*.

Purification and Storage of *Agrobacterium* Cultures

Colonies typical of *Agrobacterium*, namely smooth, white, glistening, convex and 2-4 mm in diameter on NA, as well as colonies showing a yellowish colour on the medium of Schroth *et al.* (1965) and a grey-white to fawn colour on the medium of New and Kerr (1971), were picked and streaked onto NA plates. These bacterial isolates were purified by successive streaking of single colonies on NA plates. The cultures were checked for purity by the Gram stain procedure of Conn *et al.* (1957), but with the safranin counterstain prepared according to the method from Frobisher (1963) as described by Harrigan and McCance (1966). They were then

cultured and stored on slopes of yeast-mannitol agar (YMA) (Ellis *et al.*, 1979) in McCartney bottles at 22°C in the dark.

Biotyping of *Agrobacterium* Isolates and Strains

All *Agrobacterium* isolates and strains were cultured for 24 h at 25°C on NA slopes. They were subjected to the following series of seventeen different tests, which were each repeated four times. The incubation temperature for all the biotyping tests, except the test of growth at 29, 35 and 37°C, was 25°C.

Gram reaction

The Gram staining method was that of Conn *et. al.* (1957), but with the safranin counterstain prepared according to Frobisher (1963), as described by Harrigan and McCance (1966). Slides were examined under oil immersion at 1000 X magnification.

Motility

Motility of the bacteria was examined according to the hanging drop technique described by Harrigan and McCance (1966).

Fluorescent pigment production

The production of a flourescent pigment on King's medium B was assessed as described by King *et al.* (1954).

Kovacs' oxidase test

The production of oxidase was examined using the method of Kovacs (1956).

Oxidative or fermentative metabolism of glucose

The test of Hugh and Leifson (1953) with the modified medium described by Hayward and Hodgkiss (1961) was employed.

Production of 3-ketolactose

The method developed by Bernaerts and De Ley (1963) was used, but the modified Benedict's reagent as described by Kerr and Panagopoulos (1977) was employed to detect 3-ketolactose.

Growth in presence of 2% sodium chloride

Growth in nutrient broth containing 20 g/l sodium chloride was tested according to the method described by Kerr and Panagopoulos (1977). The tubes were examined for turbidity daily for a period of 10 days.

Growth at 29, 35 and 37°C

Ability to grow at 29, 35 and 37°C was determined according to the method of Kerr and Panagopoulos (1977) using a temperature-controlled waterbath. Growth was assessed after 10 days of incubation.

Citrate utilization

The utilization of citrate was studied by the method described by Simmons (1926). Cultures were incubated for up to 7 days.

Growth and reaction in litmus milk

Growth and the reaction in litmus milk were tested as described by Kerr and Panagopoulos (1977). Incubation was for 21 days. Changes in the milk such as the production of an acid or alkaline raection or a serum zone were recorded as growth.

Acid from erythritol and melezitose

The basal medium of Hayward (1964) and the method described by Kerr and Panagopoulos (1977) were used. Cultures were incubated for 14 days.

Malonate, tartrate and propionate utilization

The tests were as outlined by Kerr and Panagopoulos (1977). That for malonate utilization was based on the production of an alkaline reaction in modified Leifson's (1933) medium. Tartrate and propionate utilization were tested on slopes of the medium of Ayers *et al.* (1919). The medium was adjusted to pH 7.0 with NaOH before autoclaving, and 0.5 ml of a separately filter-sterilized 10 g/l solution of L-tartaric acid or propionic acid, neutralized with NaOH, was added to 4.5 ml basal medium. Inoculated tubes were incubated for a period of 7 days.

Growth on selective medium for *Agrobacterium tumefaciens* biotype 1 isolates

Growth of a streak culture on the medium of Schroth *et al.* (1971) was assessed after incubation for 5 days at 25°C.

Growth on selective medium for *Agrobacterium tumefaciens* biotype 2 isolates

Growth of a streak culture on the medium of New and Kerr (1971) was assessed after incubation for 5 days at 25°C.

Growth on selective medium for *Agrobacterium tumefaciens* biotype 3 isolates

The selective medium of Brisbane and Kerr (1983) was used for the isolation and identification of biotype 3 isolates. Streaked plates were incubated for 5 days at 25°C.

Standardization of *Agrobacterium* Cell Suspensions

Preparation of cell suspensions for constructing standard graphs

Agrobacterium tumefaciens isolates 2080 and I27 were cultured on NA slopes at 25°C for 48 h. These isolates were chosen as representatives of the biotype 1 and 2 isolates. The resulting growth was suspended in 5 ml sterile distilled water. One ml aliquots of each suspension were pipetted onto five NA plates, which were incubated at 25°C for 48 h. The growth was suspended in sterile saline (8.5 g/l NaCl) and centrifuged at 12000 x g for 10 min. The cells in the pellet were washed by repeated suspension and centrifugation. The washed cells were finally suspended in 10 ml sterile distilled water.

Colorimetric determination of absorbance of cell suspensions

Tenfold dilution series of the cell suspensions from undiluted to 10^{-9} were prepared in sterile distilled water. The absorbance of suitable dilutions was determined using a Gallenkamp Model CS-200 colorimeter (Gallenkamp Co. (Pty) Ltd, London, England). Tests were conducted with a green filter (maximum transmittance at 540 nm) and a blue filter (maximum transmittance at 470 nm). The blank was dilution liquid without cells.

Dilution plate counts of cells in suspension

One ml aliquots of each tenfold dilution in sterile distilled water were pipetted onto three NA plates. Two ml of a melted 7 g/l agar solution at 45°C were poured over the inoculated Petri-dishes and mixed well with the cell suspensions. The plates were incubated at 25°C for 48 h,

after which colonies were counted using a colony counter. These counts were repeated four times.

Construction and use of a standard graph

For the two isolates, scatterplots of absorbance against cell concentration by the dilution plate method were plotted. In each case, the relationship between the two variables was also computed as a regression equation according to the least square method (Snedecor and Cochran, 1967) and represented on the graph as a regression line. To permit the determination of other *Agrobacterium* populations in suspensions accurately and rapidly by the measurement only of absorbance, the results for the two isolates were combined for the construction of a standard graph (scatterplot with regression line) in the same way as for the individual isolates.

Evaluation of Indicator Plants for *Agrobacterium tumefaciens* Biotype 1 and 2 Isolates

Seeds of tobacco (*Nicotiana glutinosa*), datura (*Datura stramonium*), broadbean (*Phaseolis vulgaris*), tomato (*Lycopersicon esculentum*) and sunflower (*Helianthus annuus*) were germinated in a glasshouse at 26°C. After 3 weeks they were transferred from seed trays to plant pots and placed in an environmental growth chamber where they were further acclimatized for another 3 days (12 h daylight, 8 000 lux, 22-25°C, 70-80% relative humidity). Carrot (*Daucus carota*) slices (discs) were also prepared, using fresh carrots which were surface sterilized by a solution of sodium hypochlorite (5 g/l solution for 10 min) after being peeled. Before inoculation, slices were placed in Petri-dishes containing water agar (15 g/l).

Agrobacterium tumefaciens biotype 1 isolates $M21_3$, $M27_6$, $M34_{11}$, $M37_{11}$, 172(a), 1887, 2086A and biotype 2 isolates C5, 39g, 46, 1671 and 2077 were grown on YMA slopes for 48 h at 25°C. Thereafter cells were harvested by suspension in sterile distilled water and the cell concentration adjusted colorimetrically to contain 1 x 10^9 cells/ml according to the standard graph. Biotype 1 isolates $M21_3$, $M27_6$, $M37_{11}$ and biotype 2 isolates 1671 and 2077 were used

to inoculate the tobacco, datura, broadbean and tomato plants. All plant inoculations were made into pre-prepared wounds (0.5 cm long) in the plant stems. Twelve replications of each treatment were made.

Biotype 1 isolates $M34_{11}$, 172(a), 1887, 2086A and biotype 2 isolates C5, 39g, 46 and 2077 were inoculated onto the sunflower seedlings and the carrot discs. Each test was replicated five times. Results were recorded every second day for a period of 42 days in the tobacco, datura, broadbean and tomato trials and for 10 days in the sunflower and carrot trials.

Evaluation of Indicator Plants for *Agrobacterium tumefaciens* Biotype 3 Isolates

Vegetatively propagated rootstocks of *Vitis* species

Fifteen cuttings were made from each of the *Vitis* rootstock cultivars stipulated under Results (Table 4). These cuttings were waxed and stored at 4°C for 3 months. They were then rooted in a mistbed, planted in plant pots and acclimatized in an environmental growth chamber (12 h daylight, 8 000 lux, 22-25°C, 70-80% relative humidity) for 10 days. The 10 best cuttings of each cultivar were selected for the evaluation experiment.

Agrobacterium tumefaciens biotype 3 isolate 2221 was cultivated for 48 h at 25°C on YMA slopes. The growth was suspended in sterile distilled water and the cell concentration adjusted colorimetrically to 1×10^9 cells/ml according to the standard graph. Superficial wounds were made on the branches of the cuttings using a sterile scalpel whereafter a loopful of the appropriate bacterial suspension was applied to each wound. Tumour development was recorded at the end of every week.

Vitis cultivar Jacquez (*Vitis aestivalis* x *Vitis cinerea* x *Vitis vinifera*) seedlings

Results obtained in the evaluation described in the previous section using the different grapevine cultivar rootstocks showed that Jacquez was extremely susceptible to crown gall

caused by *A. tumefaciens* biotype 3 isolate 2221. Subsequently a fast cultivation method was used to provide Jacquez seedlings as test plants. Seeds collected from Jacquez mother blocks at the Plant Quarantine Station, Stellenbosch, were germinated after being subjected to a cold induction period of 30 days at 4°C. The seedlings were replanted in plant pots and maintained in the controlled environment chamber (12 h daylight, 8 000 lux, 22-25°C, 70-80% relative humidity) for the duration of the experiments or tests.

Agrobacterium tumefaciens biotype 3 isolates 305, 1771, 2158, 2160, 2164, 2221, W7 and W8 were cultivated on slopes of YMA for 48 h at 25°C. Cell suspensions containing 1 x 10^9 cells/ml were prepared and the stems of seedlings inoculated in the same way as the branches in the rootstock evaluation. Three replications containing two seedlings each were used for each bacterial suspension. Tumour development was assessed every second day for 40 days.

Tobacco (*Nicotiana glutinosa*), datura (*Datura stramonium*) and sunflower (*Helianthus annuus*) seedlings

Tobacco and datura seeds were germinated in the environmental chamber (12 h daylight, 8000 lux, 22-25°C, 70-80% relative humidity) and transplanted into plant pots. When the plants reached 10-15 cm in height (3 weeks old), they were inoculated with all the biotype 3 isolates and strains, except isolate 2158, using the same wounding procedure as for the biotype 1 and 2 isolates. Five seedlings replicated three times were inoculated with each bacterial suspension. Sunflower (*H. annuus* var. *nanus* and *H. annuus* cv. *grandiflora*) seeds were also germinated in the environmental chamber (12 h daylight, 8 000 lux, 22-25°C, 70-80% relative humidity) and transplanted into plant pots. When the plants reached 10-12 cm in height, they were inoculated with the abovementioned suspensions using the same wounding procedure. Five seedlings replicated three times were inoculated with each bacterial suspension.

Serological Studies

Evaluation of media for production of *Agrobacterium tumefaciens* antigens

Three media were evaluated for the production of high yields of cells, with low amounts of extracellular polysaccharide slime, by three of the *A. tumefaciens* isolates used as antigens . The media were YMA (Ellis *et al.*, 1979), NA (Butcher *et al.*, 1980), and peptone-glycerol-yeast extract agar (PGYA) (Cambra and López, 1978). Inocula (a loopful of each) from cultures of *A. tumefaciens* isolates (TT9, 2077, and 2221) grown for 48 h on plates of YMA, NA and PGYA at 25°C were each spread evenly onto two plates of the same medium and incubated at 25°C for 48 h. The extent of extracellular polysaccharide formation was noted. The growth on each plate was suspended in 5 ml sterile water and centrifuged at 12 000 x g for 10 min. The cells were resuspended in 5 ml of sterile water, recentrifuged, resuspended in 15 ml of sterile water and shaken well by hand. The average volumes of distilled water required to dilute the suspensions to an absorbance corresponding, according to the standard graph, to 5 X 10^9 cells/ml was determined. This experiment was repeated three times.

Preparation of antigen suspensions

Antigens were prepared for injection into New Zealand rabbits and tube agglutination by growing the bacteria on NA in plates for 48 h at 25°C. The NA plates were inoculated by spreading a 0.1 ml suspension of the bacterial cells over the surface with a sterile L-shaped glass rod. Growth was suspended in 10 ml sterile saline. One half of the yield from these plates was washed five times by low speed centrifugation (10 min at 12 000 x g) and resuspended gently in sterile saline to retain the extracellular polysaccharides and flagella; these cells were designated Type A cells. The cells of the other half of each suspension were mixed with 2 mm diameter glass beads and shaken vigorously by hand for 10 min. The cells were washed three times by centrifugation at 12 000 x g for 10 min and resuspension in sterile saline. These cells, which were designated Type B cells, would have the O-antigens exposed and the flagella removed (Du Plessis *et al.*, 1979; Sutherland, 1967). Both groups of cells

suspended in 20 ml of sterile saline were filtered through a sintered glass filter of pore size 40-50 um to remove cell aggregates. The cell suspensions were diluted to give an absorbance corresponding to a cell concentration of approximately 2 x 10^9 cells/ml according to the standard graph. These antigen suspensions were preserved by the addition of 400 g/l formaldehyde solution to a final concentration of 3 g/l and subsequent storage at 4°C.

For Ouchterlony immunodiffusion studies antigens were prepared from both A and B type antigens in the following six different ways:

(i) Suspensions with untreated cells as the antigen were prepared as described above, except that the cell concentration was 1 x 10^9 cells/ml.

(ii) Suspensions with heat-treated cells as the antigen were produced by heating *A. tumefaciens* cell suspensions prepared as in (i) in a waterbath at 100°C for 60 min.

(iii) Phenol-treated cells were prepared as described by Vruggink and Geesteranus (1975). This phenol treatment entailed the addition of one drop of saturated phenol solution into every 1-2 ml of antigen suspension, whereafter air was blown through it for 1 min.

(iv) Ultrasonically disintegrated cells were prepared by treating different 5 ml aliquots of bacterial suspensions containing 1 x 10^9 cells/ml for 5, 10 and 15 min, respectively, in a Labsonic 9100/1 ultrasonic disintegrator (Labline Instruments, Illinois, USA). The cells were suspended in sterile distilled water for the ultrasonic treatment, as cells in a liquid growth medium or saline are usually more resistant to disintegration (Sutherland, 1967). The disintegrated cells were used as antigens without further treatment.

(v) Diffusible antigens were obtained by centrifuging nutrient broth cultures of the bacteria (grown at 25°C for 48 h) for 10 min at 12 000 x g. The supernatant liquid was tested for diffusible antigens.

(vi) Trichloroacetic acid (TCA) extraction to obtain whole "Boivin" somatic antigen was performed according to the procedure of Boivin and Mesrobeanu (Staub, 1967). Isolates D3, TT9, $M34_{11}$, 2080, 2077, 305 and 2221 were grown on NA plates for 48 h at 25°C. The cells were suspended in 7 ml sterile saline, centrifuged at 12 000 x g for 10 min and washed by twice repeating the suspension-centrifugation procedure. The final cell pellets were suspended in five times their mass of water at 0°C. An equal volume of 1 N trichloroacetic acid at 0°C was

added and the mixture stirred for 3 h at 0-4°C. Cell debris was removed by centrifugation at 12 000 x g for 30 min, and the supernatant dialyzed for 48 h against running tap water to remove all trichloroacetic acid. To each 100 ml of solution, 5 ml of 95% (v/v) ethanol at 4°C was added. The resultant precipitate was removed by centrifugation at 12 000 x g for 30 min. A further 35 ml of cold ethanol (4°C) was added to 150 ml of the supernatant, which was then left for 12-16 h at 4°C. The somatic antigen which precipitated at this stage was removed by centrifugation at 12 000 x g for 30 min, washed with ethanol and dried under vacuum. The somatic antigen fractions were suspended in 5 ml sterile saline before use. Tube agglutination tests were conducted with the discarded bacteria to determine whether their somatic antigenic determinants had been removed.

Preparation of antisera

Antisera were prepared against cells of isolates TT9, D3 and 2077 still containing their extracellular polysaccharides (type A), as well as against cells of three biotype 1 isolates (TT9, $M34_{11}$, 2080), two biotype 2 isolates (D3, 2077) and two biotype 3 isolates (305, 2221) devoid of extracellular polysaccharides (type B). Six-week-old New Zealand rabbits were injected intravenously with these antigens (one rabbit per antigen). Thirty ml of blood were obtained from each rabbit prior to the injections to obtain pre-immune serum.

The left ear of each rabbit in the area of the marginal vein was plucked by hand to free the area of hair (shaving these areas usually leaves a raw area which is further irritated by the application of a disinfectant). These areas were then disinfected with a 70% (v/v) ethanol solution. The rabbits were immunized by intravenous injection on four consecutive days with, respectively, 1, 2, 3 and 3.5 ml antigen suspension containing approximately 1 x 10^9 cells/ml in sterile saline (Loos and Louw, 1964). Injections were performed with 10 ml sterile glass syringes and sterile stainless steel no. 22 needles. Injections began at the top of the ear and progressed towards the base, with the needle inserted in the direction of blood flow.

The rabbits were bled 14-16 days after the last injection. All food was withheld for 24 h prior to bleeding. Blood was obtained after plucking the hair in the area of the marginal vein of the right ear. The area was disinfected with 70% (v/v) ethanol and xylene applied with cotton wool swabs for vein dilation. Petroleum jelly was spread over the area where the incision was to be made, to allow all blood to flow off the ear surface. A cut perpendicular to the ear vein was made with a sterile scalpel, and 10 ml blood collected for a trial test of antibody titre. If the antibody titre proved to be suitable (640 and above), 60 ml blood was collected on each of two consecutive days. The collected blood was allowed to clot at 27°C for 3 h, whereafter the clot was cut into small pieces and the blood left overnight in the refrigerator at 4°C. The straw-coloured liquid (serum) was removed with a sterile Pasteur pipette. To remove residual red blood cells from the serum it was centrifuged for 10 min at 12 000 x g. All the antiserum obtained from a particular animal was pooled, and 0.25 g/l sodium azide added as a preservative. The antisera were decanted into small glass vials in 3 ml aliquots and preserved by freezing at -10°C.

Initial trials of serological test techniques and specificities of antisera

Two different variations of the Wasserman tube agglutination method were tested. In the first procedure the test tubes were kept in a waterbath at 37°C for 3 h. In the second procedure the tubes were incubated in a waterbath at 47°C for 4-5 h and kept overnight at 4°C. Ordinary 0.01 M phosphate buffered saline (PBS) at pH 7.4 (0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4.12H_2O$, 8.5 g NaCl, 0.2 g KCl, 0.2 g NaN_3, 1 l distilled water) and normal serum were included as controls. The different antisera were diluted in a two-fold series from 1:10 to 1:5 120 (final test concentrations) in PBS. To determine a suitable antigen concentration, the different antigens (types A and B) were diluted in a two-fold series from 4 x 10^9 to 1.95 x 10^6 cells/ml. Each diluted antigen suspension was also divided in half; one part was not treated and the other half was heated at 100°C for 60 minutes.

Different Ouchterlony immunodiffusion test procedures were also tested. Petri-dishes were each filled with 30 or 20 ml gel as described by Humphrey and Vincent (1965) with Orange G

dye added according to Erasmus *et al.* (1974). Two different central (antiserum) well sizes were employed, namely, 6- and 8-mm-diameter wells. They were surrounded by 6 hexagonally placed 6-mm-diameter wells at distances of 6 and 5 mm, respectively. Two different incubation temperatures were also used, namely 4 and 27°C. Antigen preparations tested comprised type A and B antigens subjected to the six different treatments described in the section 'Preparation of antigen suspensions.' The method(s) producing the best result(s) were used in all further serological tests.

Wassermann agglutination tests

Equal volumes (1 ml) of antigen and antiserum were pipetted into small glass test tubes (7.5 mm x 0.8 mm). Antisera diluted in sterile saline to give two-fold dilutions ranging from 1:20 to 1:5 120 were tested against antigens at a concentration of 5 x 10^8 cells/ml or other concentrations found to be more efficient (yielding higher titres with less antigen) in the initial tests. These antiserum and antigen values were the final dilutions and concentrations after the antiserum was mixed with the antigen. The tubes were kept in a waterbath at 47°C for 5-6 h, then left overnight at 4°C in a refrigerator.

Heated cell suspensions were used to show only somatic (O) reactions and untreated cell suspensions to show both flagellar (H) and O reactions. According to Lucas and Grogan (1968), the heat treatment can also show, by a reduction of the O-reaction titre, the presence of thermolabile O-antigens which produce species-specific reactions. Pre-immune serum and saline controls were included in the tests which were repeated three times. Reactions were observed against a black background with the tubes illuminated by a straight beam from a light source situated behind and to one side of them. Flagellar reactions (H) produced floccular precipitates after 15-30 min, and somatic reactions more compact granular flakes or clumps over a longer period of time.

Ouchterlony immunodiffusion tests

The gels described by Humphrey and Vincent (1965) were used, with Orange G dye added according to Erasmus *et al.* (1974) for better observation of precipitin bands. Twenty ml of gel were poured into 9-cm-diameter disposable plastic Petri dishes. The dishes were flat-bottomed, and the wells did not need to be sealed off. The well pattern consisted of a central 8-mm-diameter well, surrounded by six hexagonally placed 6-mm-diameter wells. Phenol-treated antigen suspensions of 5 x 10^8 cells/ml were placed in the peripheral wells, and different antiserum dilutions (1/4, 1/8 and 1/16) in the central wells. Every second peripheral well contained the homologous antigen. Controls of pre-immune serum, and sterile distilled water were included. Incubation temperature was 27°C. These Ouchterlony plates were kept in humid stainless steel Petri dish containers and examined periodically for precipitin bands between the antiserum and antigen wells. Precipitin bands were seen more clearly if the Petri dishes were illuminated from the bottom. The precipitin bands were photographed from above with a special fluorescent ring light situated beneath the Petri dishes.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA method of Clark and Adams (1977), with a few variations, was used (Fig. 2). The complete procedure was as follows:

Isolation and purification of gamma-globulin. A 3 ml sample of each antiserum was diluted to 10 ml with distilled water. Ten ml saturated ammonium sulphate solution were then added. The mixtures were stirred very slowly for 3 h, then left at room temperature for 2-3 h. The resultant precipitates were collected by centrifugation at 12 000 x g for 10 min. The pellets were dissolved in 2.5 ml 0.02 M PBS and the solutions desalted by exclusion chromatography using Sephadex G-25 PD 10 columns (Pharmacia Ltd., Uppsala, Sweden). The columns were connected to a Type 8303 A detector unit and a Uvicord II 8300 control unit of a Uvicord II ultraviolet absorptiometer, (LKB Instruments Ltd., Seldon, Surrey, England). Graphs were produced on a Linear Beckman Recorder (Beckman Instruments Inc., Fullerton, California,

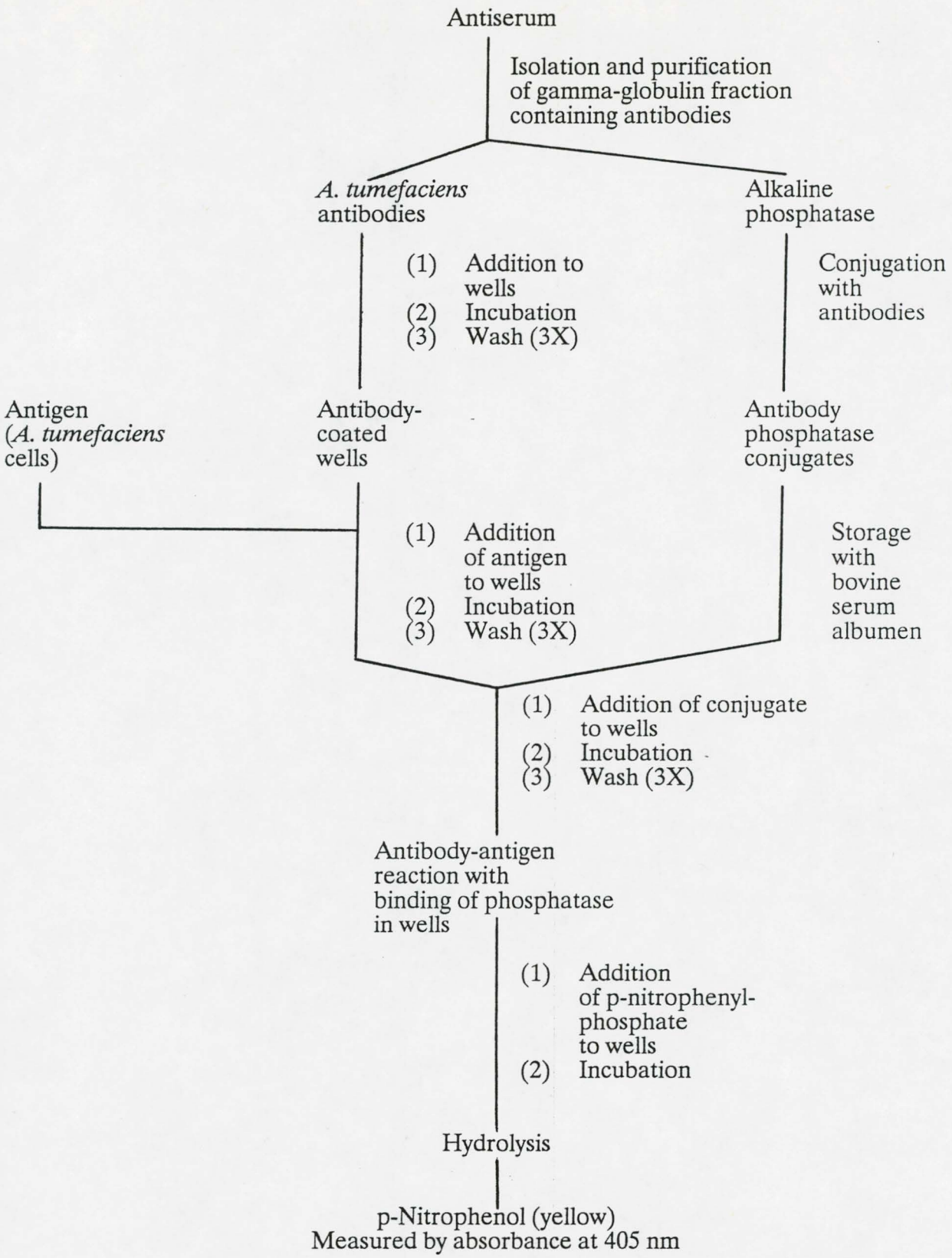


Fig. 2. Outline of the microplate ELISA technique for the detection of *A. tumefaciens* antigen-antibody reactions

USA) operating at 10 mV and 1.27 cm/min. The fractions showing the major peak on these graphs were further purified by passage through columns of Pharmacia DEAE-Sephacel cellulose with wet particle size 140-150 µm. These columns were treated in the same way as the Sephadex G-25 columns, and again only the fractions showing the major peak were collected. The gamma-globulin concentrations were adjusted with PBS to approximately 1 mg/ml (E_{280} = 1.4) using a Model 100-60 Hitachi UV spectrophotometer (Hitachi Ltd., Tokyo, Japan). The gamma-globulin preparations were stored in small glass vials at -10°C.

Conjugation of alkaline phosphatase with gamma-globulin. Two mg of Pharmacia alkaline phosphatase were dissolved in each 1 ml gamma-globulin preparation, which was then dialyzed four times against 1 l PBS at 4°C. Glutaraldehyde was added according to the method of Avrameas (1969) to give a final concentration of 5 µg/ml, and the mixture incubated at 4°C for 4 h. The glutaraldehyde was removed by dialysis against four changes of PBS (each 1 l). The conjugate preparation was supplemented with 10 µg/ml bovine serum albumin and stored at 4°C in glass vials.

Coating of the microplates. The concentrations of gamma-globulin used for coating the wells were 10, 1.0 and 0.1 µg/ml. The Bios Titertek plates or Linbro EIA microtitre plates (Bios Laboratories (Pty.) Ltd., Sandton, R.S.A.) contained 96 flat-bottomed wells (Fig. 3A,B). Eighteen wells of each plate tested were coated (200 µl) with each of the different concentrations of purified gamma-globulin concentration mentioned above with the use of the Titertek Autodrop (Fig. 3C) and incubated at 30°C for 3 h in a moist incubator. The gamma-globulin was made up in coating buffer (0.05 M sodium carbonate at pH 9.6). The plates were gently washed three times with a stream of PBS-Tween 20 (PBS containing 0.05 ml/l Tween 20) and shaken dry. To establish the optimum antigen concentration for the assay, 200 µl of type B antigen suspension with approximately 1 x 10^9, 1 x 10^7 or 1 x 10^5 cells/ml were placed in the wells, after which the plates were incubated in a moist chamber for 12-16 h at 6°C. The plates were then gently washed three times with a slow stream of PBS-Tween 20, and shaken dry. Enzyme-linked gamma-globulin (200 µl) at dilutions of 1/200, 1/400, and 1/800 in PBS-Tween 20 containing 2 g/l ovalbumin was placed in the wells. The plates were again incubated at 30°C

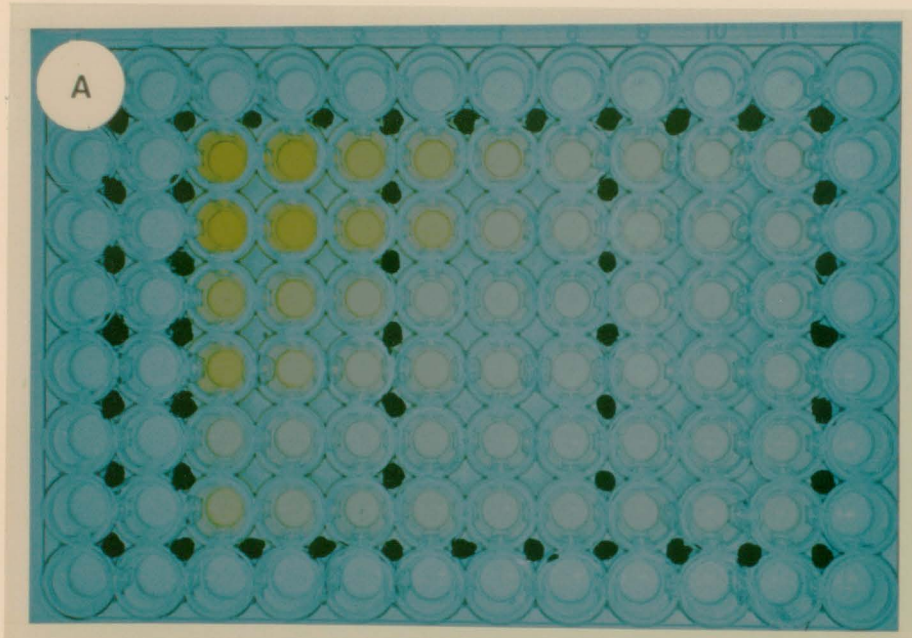
Fig. 3. Microtitre plates and apparatus used in the ELISA test procedures

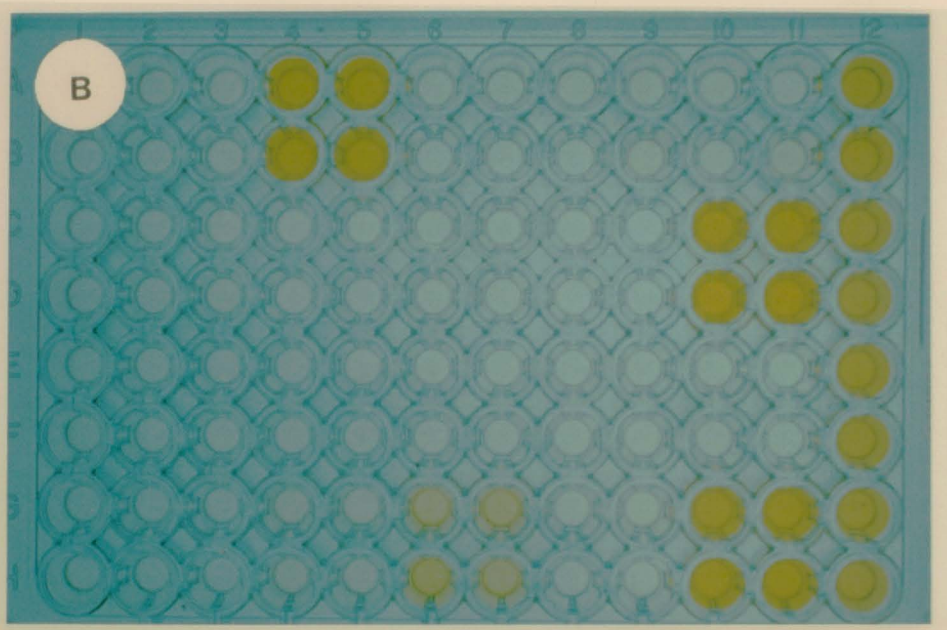
A. Determination of suitable reactant concentrations for ELISA with anti-D3 gamma globulin. The outer row of wells was not used as artifacts resulting from temperature fluctuations caused variable A_{405} readings (side effect). The yellow colouration of the enzyme-substrate reaction was strongest in the upper left region at the highest concentration of reactants (10 ug/ml coating gamma globulin, 1 x 10^9 bacteria/ml, 1/200 dilution of enzyme-labelled gamma globulin).

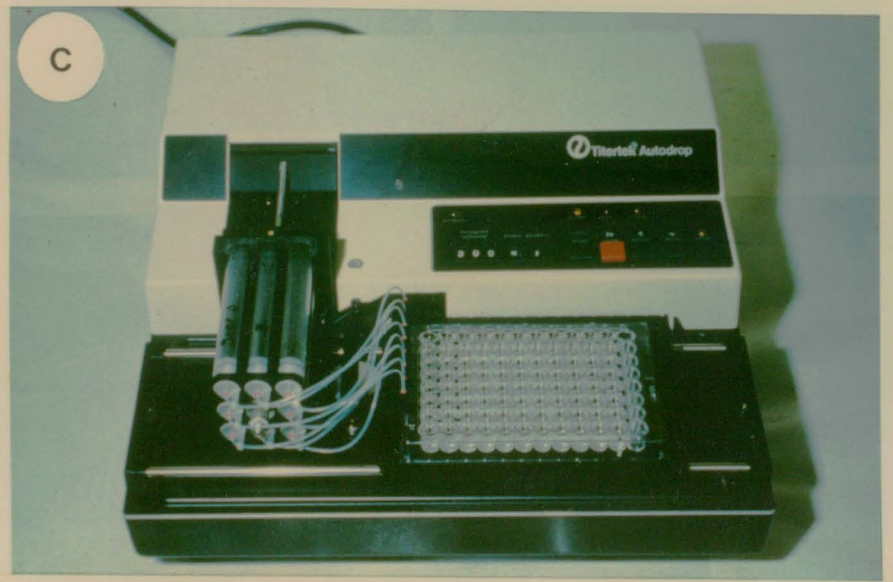
B. Testing of different heterologous cell preparations against 10 ug/ml coating anti-2077 gamma-globulin, 1/200 enzyme-linked anti-2077 gamma-globulin and 1 x 10^9 bacterial cells/ml. Vertical row 1 wells contained the PBS control and vertical row 12 wells homologous cells (isolate 2077) as control antigen. Wells A 4, 5 and B 4, 5 show the reaction with isolate I27, wells C 10, 11 and D 10, 11 the reaction with isolate C5, and wells G 10, 11 and H 10, 11 the reaction with isolate 41B. All these reactions produced an intense colour. A weaker colour reaction developed with antigen 47 in wells G 4, 5 and H 4, 5.

C. The Titertek Autodrop apparatus for administrating the correct amounts of each reactant in each microtitre well. The washing of the microtitre plates in PBS-Tween 20 was done by hand.

D. The Titertek Multiscan apparatus used for the determination of the ELISA A_{405} values.







for 3 h in a moist chamber, gently washed three times in a slow stream of PBS-Tween 20 and shaken dry.

Determination of phosphatase fixed in wells. Diethanolamine buffer (100 ml/l diethanolamine adjusted to pH 9.8 with HCl) containing 1 mg/ml p-nitrophenylphosphate (Sigma, St. Louis, Missouri, USA) was placed in each well (300 µl). The development of a yellow colour was observed visually (Fig. 3A,B) and by means of a Bios Titertek Multiscan (EFLAB OY, Helsinki, Finland) (Fig. 3D) after 2 and 4 h at 22°C, as well as after incubation overnight at 4°C. The Bios Multiscan gave readings of absorbance at 405 nm (A_{405}).

After suitable concentration-dilution combinations were determined for the homologous antigen-antibody mixtures, these concentrations and dilutions were used in similar assays involving heterologous antigen-antibody combinations.

In Vitro Inhibition of *Agrobacterium tumefaciens* Isolates by Bacteriocogenic Strains *Agrobacterium radiobacter* K84 and *Agrobacterium tumefaciens* D286

Agrobacterium radiobacter K84 and *A. tumefaciens* D286 were tested for their ability to inhibit the available pathogenic *A. tumefaciens* isolates. *Agrobacterium tumefaciens* D286, isolated from *Eucalyptus* sp., was a bacteriocin-producing pathogen that had spontaneously lost its pathogenicity but not the ability to secrete a bacteriocin (Hendson *et al.,* 1983). Inoculation of this strain onto tobacco and datura indicator plants confirmed its non-pathogenicity.

The two bacteriocinogenic isolates were grown on YMA slopes for 48 h at 25°C. A loopful of each strain was transferred to 30 ml liquid basal medium of Stonier (1956), to which 0.01 g/l yeast extract and 200 µg/l biotin had been added (SM), YMA without agar and the modified soil extract agar (SEA) of Bunt and Rovira (1955) without agar. The cultures were incubated with shaking at 100 rpm for 48 h at 25°C. Duplicate plates of each of the three media were poured into glass Petri-dishes for each pathogen to be tested. One ml of each bacteriocinogenic strain from each of the three liquid cultures was placed in a test tube

containing 2 ml of the corresponding medium plus 9 g/l Difco Bacto agar and 0.1 ml amounts were placed in the middle of the prepoured plates of corresponding agar media. After incubating the plates at 25°C for 72 h, the Petri dishes were inverted and filter paper disks placed within the lids. The filter paper discs were saturated with chloroform ($CHCl_3$) and the dishes closed for 60 min. The inverted bottoms of the dishes were then slanted on one side of the lids to allow the chloroform to evaporate.

In the meantime, a loopful of each pathogenic strain had been inoculated into 10 ml of each of the liquid media and incubated at 25°C for 72 h. From these cultures, 0.5 ml volumes were inoculated into 2.5 ml 0.1 M phosphate buffer of pH 7 containing 7 g/l Difco Bacto agar. The suspensions were rotated well, then used to overlay the chloroform-treated plates on which the bacteriocinogenic agrobacteria had been cultured. The overlaid plates were rotated well before the overlay agar solidified. The plates were incubated at 25°C and inhibition zones recorded daily. This experiment was repeated three times.

Selected colonies of *A. tumefaciens* biotype 1 isolates TT9, 172(a), 198, 1465, 2086A and *A. tumefaciens* biotype 2 isolates A1, and 39m which developed inside and outside the inhibition zones were tested for pathogenicity. The reason for this study was the discovery by Engler *et al.* (1975) and Kerr and Htay (1974) that resistant non-pathogenic *A. tumefaciens* colonies developed in the presence of agrocin 84 *in vitro*; however, Cooksey and Moore (1982b) and Süle and Kado (1980) found that a number of resistant strains were still pathogenic. The colonies were picked off the plates and purified by four successive streakings of single colonies. The streak plates were incubated at 25°C for 48 h. The final streak plates, when the cultures were considered pure, were flooded with 5 ml sterile distilled water and bacterial suspensions with an absorbance equivalent to that of 1 x 10^9 cells/ml were prepared using the standard graph. These suspensions were inoculated onto 3-week-old tobacco and datura seedlings which had been wounded in the stems with a sterile scalpel. Two drops of each suspension were placed on four tobacco and four datura seedlings. The plants were grown further in a glasshouse and examined for tumour development twice a week for a 6-week period. The

picking of colonies for the investigation of their pathogenicity was repeated from three different sets of plates from replicate experiments.

In Vivo Inhibition of *Agrobacterium tumefaciens* Isolates by Bacteriocinogenic Strains *Agrobacterium radiobacter* K84 and *Agrobacterium tumefaciens* D286

Growth conditions for test plants and bacteria

The *in vivo* inhibition (biological control) of available pathogenic *A. tumefaciens* isolates by *A. radiobacter* K84 and *A. tumefaciens* D286 was tested in glasshouse experiments. The experimental variables were the different pathogens, bacteriocinogenic strains, host plants and times of application of inocula. The glasshouse was north-facing with temperatures inside set at 15°C night temperature and 26°C daytime temperature. The relative humidity was set between 55 and 60% and plants were irrigated every second day.

The test plants were 3-week-old tobacco and datura seedlings. All *Agrobacterium* isolates were initially grown on YMA slants at 25°C for 48 h. The cells were suspended in 3 ml sterile distilled water and inoculated onto NA plates, which were incubated at 25°C for 48 h. The growth was suspended in 5 ml sterile distilled water and the cell concentration adjusted colorimetrically, according to the standard graph, to 1 x 10^9 cells/ml. Subsequently, the bacteria were inoculated onto the test plants and results recorded as indicated in the following sections.

Application of pathogenic isolates 24-30 h after bacteriocinogenic strains

Two drops of a bacteriocinogenic strain were placed onto a wound made in the stem of each tobacco and datura test plant. After 24-30 h, two drops of the pathogen were placed on each wound. The 24-30 h difference in time of inoculation would give the first applied strain time to colonize the attachment sites on the plant host cell walls (Cooksey and Moore, 1982a). Controls inoculated with distilled water, a bacteriocinogenic strain, and a pathogenic strain

were included. The treated seedlings were placed in a glasshouse and tumour development recorded after 30 days. The test was replicated four times.

Application of bacteriocinogenic strains 24-30 h after pathogenic isolates

This experiment was conducted as described in the previous paragraph but with the bacteria applied to the test plants in the reverse order.

Simultaneous application of pathogenic isolates and bacteriocinogenic strains in different ratios

Bacteriocinogenic strains *A. radiobacter* K84 and *A. tumefaciens* D286 were tested separately against each pathogen at different ratios. The ratios of each pathogen to each bacteriocinogenic strain were 10:1, 3:1, 1:1, 1:3 and 1:10. The experiments were conducted further as described above, except that two drops of each mixed suspension of pathogen and bacteriocinogen were added to a wound. The experiments are shown diagrammatically in Fig. 4.

Investigation of the possible synergistic action of *Agrobacterium radiobacter* K84 and *Agrobacterium tumefaciens* D286 against different pathogenic isolates

Bacteriocinogenic strains *A. radiobacter* K84 and *A. tumefaciens* D286 were mixed in a 1:1 ratio and the resulting suspension was mixed in different ratios with each pathogen (Fig. 5). Two drops of the inoculum were placed onto a wound on the indicator plant (tobacco and datura).

Recording of tumour development and calculation of inhibition by bacteriocinogens

All plants were cut off at crown level and tumours (if present) were removed. Both plants and tumours were weighed separately. The masses of each were recorded on computer sheets and a biological control index (BCI) suitable for statistical analysis was computed as follows:

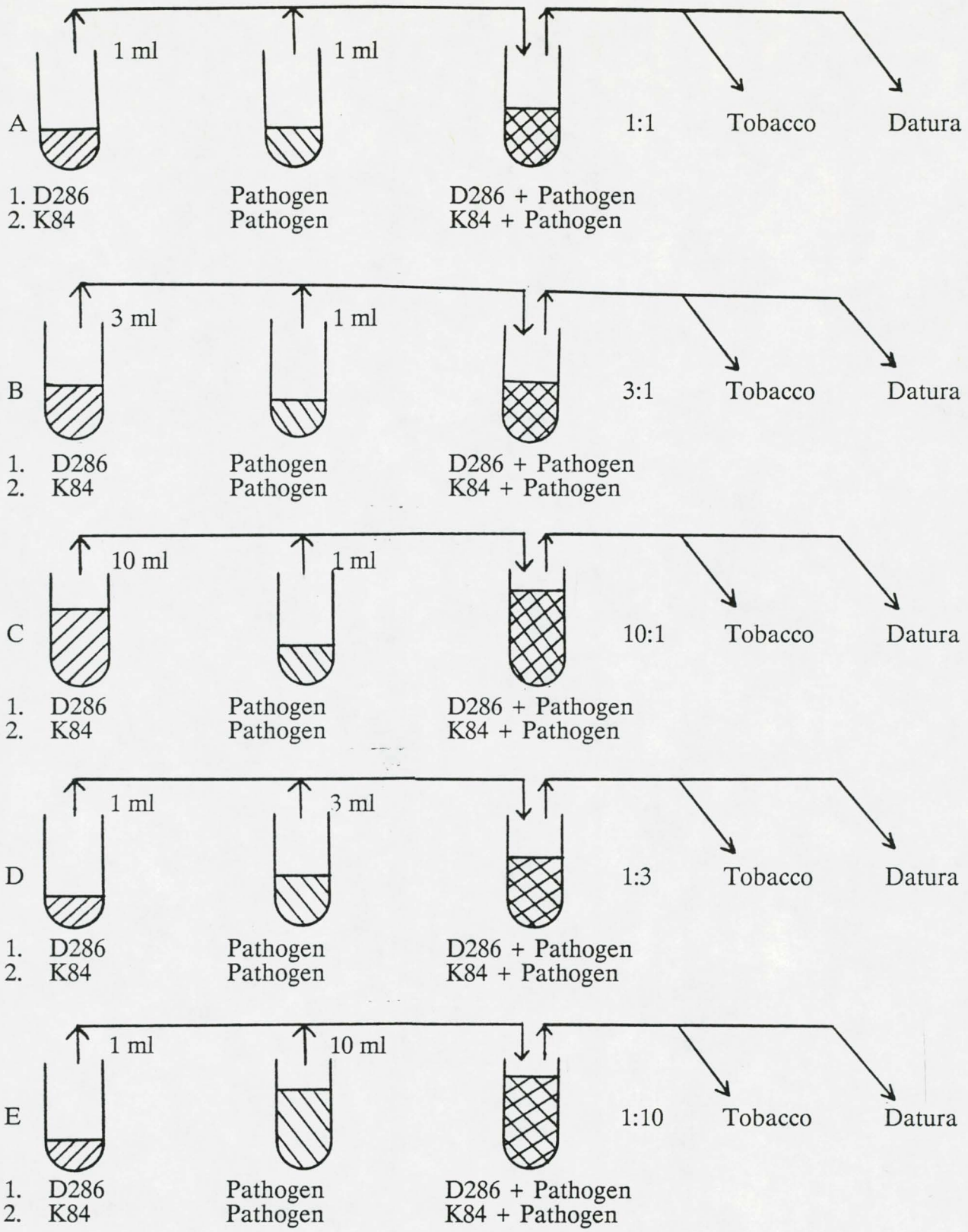


Fig. 4. Simultaneous application to test plants of bacteriocinogenic *A. radiobacter* K84 or *A. tumefaciens* D286 with different pathogenic *A. tumefaciens* strains in different ratios. The ratios were A, 1:1; B, 3:1; C, 10:1; D, 1:3 and E, 1:10.

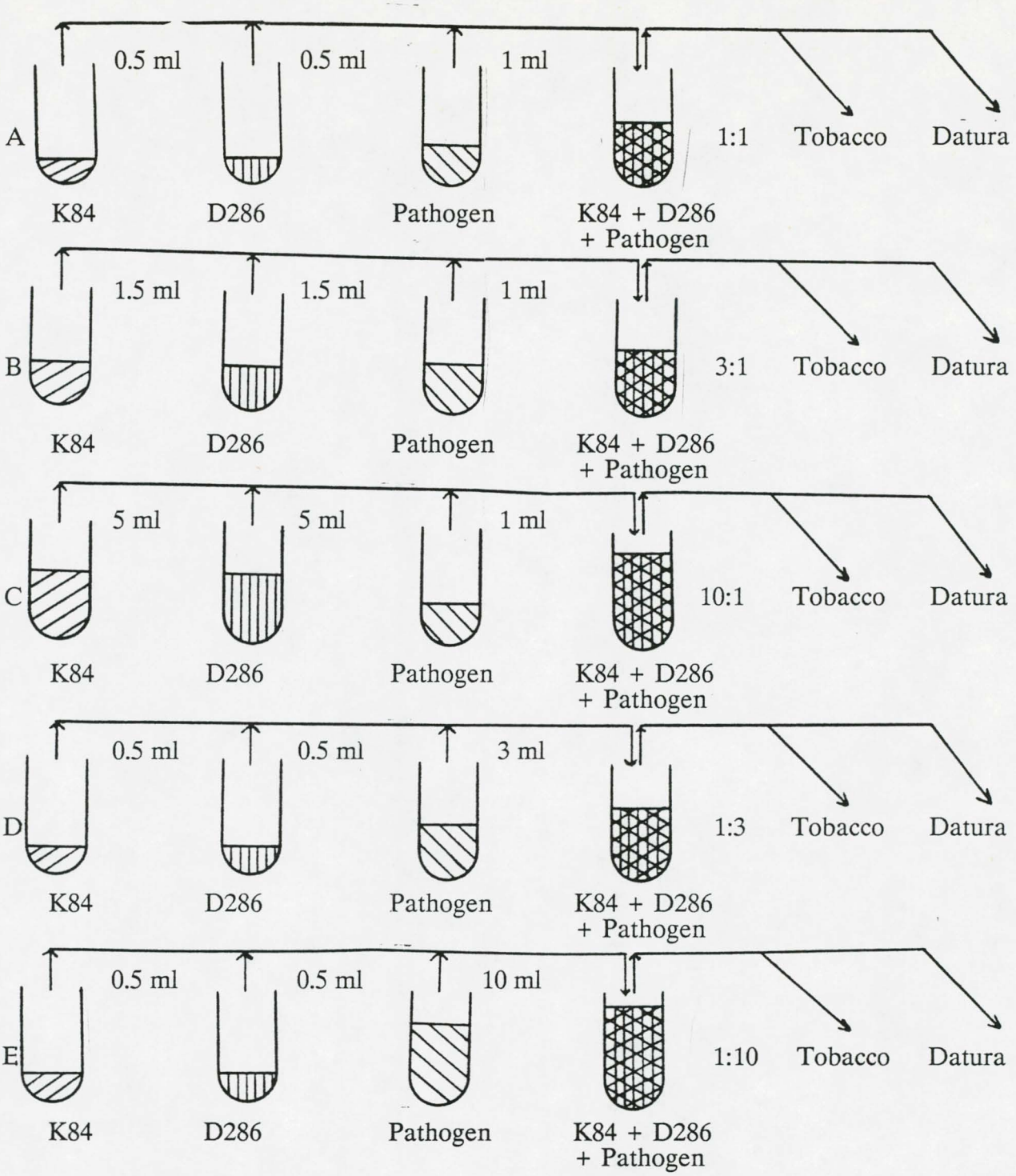


Fig. 5. Simultaneous application to test plants of different ratios of pathogenic *A. tumefaciens* strains and a 1:1 mixture of *A. radiobacter* K84 and *A. tumefaciens* D286. The antagonist:pathogen ratios were A, 1:1; B, 3:1; C, 10:1; D, 1:3 and E, 1:10.

BCI = wet plant mass/wet tumour mass

The plant mass was divided by the tumour mass as the computation was much easier with the resulting relatively large number than it would be with a fraction containing decimal noughts. Thus the larger the BCI, the smaller the tumour in relation to the mass of the test plant. Where no tumours were present a score of 1/100 of the mean mass of the control plants without tumours was recorded to avoid infinity values during the computation of the BCI values. Very small tumours which were too small to weigh were scored as 0.004 g.

The results were subjected to analysis of variance and levels of significance in pairwise comparisons determined by the Bonferroni test (Neter and Wasserman, 1974). The value given for this test is the simultaneous significant P value of comparisons of all pairs of means.

Inhibition of *Agrobacterium tumefaciens* and Tumour Development by Chemical Agents

D-Glucosamine

***In vitro* sensitivity of pathogenic agrobacteria to D-glucosamine.** A 0.5 ml aliquot of a filter-sterilized (0.2 um membrane filter) 0.1 M D-glucosamine solution was mixed with 0.5 ml of melted 7.0 g/l Bacto Difco agar, and 0.1 ml amounts were pipetted onto the middle of YMA and NA plates. The plates were kept at room temperature (22°C) for 48 h to allow the D-glucosamine to diffuse into the medium. *Agrobacterium tumefaciens* biotype 1 isolates TT9, K14, K28, 1895 and *A. tumefaciens* biotype 2 isolates 39m, 70 and 2077 (0.5 ml of a suspension of each pathogen at a cell concentration of 1×10^9 cells/ml) were each mixed with 2.5 ml 7.0 g/l Difco Bacto agar and overlaid on the two sets of D-glucosamine-containing plates, which were then rotated well and incubated at 25°C for 48 h. Bacterial growth and the presence of inhibition zones were checked daily for 7 days. This experiment was repeated four times.

Treatment of tumours on tobacco and datura indicator plants with D-glucosamine. Inhibition of tumour development by D-glucosamine was tested according to a method based on that described by Richardson *et al.* (1976). The *A. tumefaciens* isolates used in the *in vitro* study were cultured on slopes of YMA at 25°C for 48 h. Growth was suspended in 3 ml sterile distilled water and pipetted onto NA plates which were incubated at 25°C for 48 h. The resultant growth on these plates was suspended in 5 ml sterile distilled water and cell concentrations adjusted colorimetrically to 1 x 10^9 cells/ml, according to the standard graph. Three-week-old tobacco and datura seedlings were wounded on the stems with a sterile scalpel and two drops of pathogen suspension applied to the wounds. The plants were kept in a controlled environment growth chamber (12 h daylight, 8 000 lux, 15-25°C, 70-80% relative humidity) and tumour development was monitored on a daily basis. As soon as most tumours were 0.5 cm in diameter, a solution of 0.1 M D-glucosamine was applied directly onto all tumours every 24 h. Controls inoculated with the pathogens but not treated with D-glucosamine and distilled water controls were also included. Further tumour development and regression or disappearance of tumourous tissue was investigated every 24 h for a period of 30 days.

Extract of the alkaloid-producing weed *Hypericum perforatum*

Preparation of the *Hypericum perforatum* **extract.** An extract of this weed was made according to a method based on that described by Popova and Cikova (1978). They found the extract to have an inhibitory effect on *A. tumefaciens in vitro* on potato agar plates as well as on the further development of established tumours on stems of *D. stramonium*.

Hypericum perforatum plants were collected along the Helshoogte Pass, Stellenbosch. The weeds were planted in 120 mm diameter plastic pots containing potting soil and kept in a glasshouse. After 2 weeks, 250 g of alkaloid-containing leaf material were harvested and homogenized in 200 ml sterile distilled water in a Waring blender. The homogenate was passed through a cheese cloth and filtered through filter paper to yield a clear extract,

whereafter the filtrate was filter-sterilized (0.2 um membrane filter). The *H. perforatum* extract was stored at 4°C.

***In vitro* sensitivity of pathogenic agrobacteria to the *Hypericum perforatum* extract.** The test was performed in the same way as the test described by Popova and Cikova (1978) using *A. tumefaciens* biotype 1 isolates $M21_3$, K17, 57 and T-37 and *A. tumefaciens* biotype 2 isolates A1, C5, 38m and 46. Bacterial growth and inhibition zones were recorded daily for 20 days.

Treatment of tumours on indicator plants with *Hypericum perforatum* extract. The test was conducted on tumours caused by the eight *A. tumefaciens* isolates used in the D-glucosamine study. Tobacco and datura seedlings which had been wounded and inoculated with the pathogenic agrobacteria were left in a glasshouse until tumours were visible and approximately 0.5 cm in diameter. The tumours on the plants were then treated daily with undiluted *H. perforatum* extract which was applied either directly onto the tumour surface with a small brush, or by injecting 0.05 ml amounts into the tumour at 10 different sites. The plants were kept in the controlled environment growth chamber and tumour regression or absence thereof recorded every second day for 30 days. This test was repeated three times.

Gallex

***In vitro* sensitivity of pathogenic agrobacteria to Gallex.** All *A. tumefaciens* isolates listed in Table 1 were cultured on YMA slants. After incubation for 24 h at 25°C the cells were collected in sterile distilled water and diluted to contain 1 x 10^9 cells/ml according to the standard graph. Filter paper discs (1 cm in diameter) were soaked in the Gallex for 15 seconds and placed in the middle of NA plates. The plates were placed on the benchtop at room temperature (22°C) overnight to allow the potentially active chemicals in Gallex to diffuse into the medium.

From the *A. tumefaciens* cultures, 0.5 ml volumes were pipetted into 2.5 ml 0.1 M phosphate buffer of pH 7 containing 7 g/l Difco Bacto agar. These test tubes were rotated well and used

to overlay the Gallex-containing plates. Any inhibition area surrounding the Gallex-containing paper disc would be a positive result. This test was repeated three times.

Treatment of tumours on indicator plants with Gallex. *Agrobacterium tumefaciens* biotype 1 isolate TT9 and biotype 2 isolates D3 and 2077 were cultured on YMA slants at 25°C for 48 h, collected in sterile distilled water and the concentration adjusted colorimetrically, according to the standard graph, to 1 x 10^9 cells/ml. Three-week-old tobacco and datura seedlings were inoculated in the controlled environment growth chamber (12 h daylight, 8 000 lux, 15-25°C, 70-80% relative humidity) with suspensions of the three pathogens. Each pathogen was inoculated onto six tobacco and six datura plants. After tumour development to approximately 0.5 cm in diameter, the Gallex liquid was applied with a paintbrush to the tumours on three tobacco and three datura plants. The entire tumour area, as well as some of the healthy tissue surrounding the tumour, was well covered with Gallex. The tumours on two tobacco and two datura plants were completely removed with a sterile scalpel. Gallex was applied with a paintbrush to the wounds, and on the healthy surrounding tissue. The rest of the plants (one tobacco and one datura) served as positive controls. Any regression of tumour material was recorded where the Gallex was applied to the entire tumour and would indicate that Gallex caused the tumour to disappear. On the other hand, any resulting new tumourous outgrowths reappearing where tumours were removed were recorded and would indicate that Gallex was ineffective in controlling new and developing crown gall tumours.

Heat-Sensitivity of *Agrobacterium tumefaciens* Isolates and Host Plant (Peach) Seedlings

Heat-sensitivity of *Agrobacterium tumefaciens* isolates

Agrobacterium tumefaciens biotype 1 isolates 1465, 1895 and 2080, biotype 2 isolate 2077 and biotype 3 isolate 2221 were cultured on YMA slopes at 25°C for 48 h. Suspensions were prepared in PBS and the cell concentration adjusted colorimetrically to 2 x 10^9 cells/ml according to the standard graph. Five ml of each *A. tumefaciens* suspension in tubes were treated for 0-80 min (with intervals of 10 min) at temperatures over the range 20-60°C (with

intervals of 10°C). An untreated control was included. After each heat treatment the tubes were cooled under running tap water for 15 min. After the cooling process, 0.5 ml aliquots of bacterial suspension from each treatment were streaked onto two NA plates with a sterile L-shaped glass rod and incubated at 25°C for 48 h. Bacterial growth (colony formation) was investigated after 48 and 72 h. Three-week-old tobacco and datura seedlings were wounded on the stems, and two drops of each of the suspensions, including the control, were inoculated onto these areas. The plants were incubated in a controlled environment growth chamber (12h daylight, 8 000 lux, 15-25°C, 70-80% relative humidity) and the presence or absence of tumours investigated every 48 h for 30 days. This experiment was repeated three times.

Heat treatment of peach (*Prunus persica* cv. Kakamas) seedlings

A hot water treatment of 50°C for 15 min is generally used to rid plant material of fungal and viral pathogens (Raychaudhuri and Verma, 1977; Von Broembsen and Marais, 1978). Thirty two-year-old dormant peach seedlings were uprooted and placed in water of different temperatures ranging from 20°C to 60°C (with a 10°C interval) for 15 min. Five seedlings were treated at each temperature. After the treatment the seedlings were replanted in potting soil and left in a controlled environment growth chamber (12h daylight, 8 000 lux, 15-25°C, 70-80% relative humidity). Plant growth was assessed every 24 h for 20 days. This experiment was repeated three times.

RESULTS

Agrobacterium tumefaciens Isolates and Strains

As can be seen from Table 1, *A. tumefaciens* isolates of all three biotypes and from various hosts were included among the South African isolates. Figure 6 indicates that they were from many different localities which included both agricultural and urban areas. The *A. tumefaciens* isolates found in urban areas were usually from nurseries. Isolates obtained in agricultural areas were from stonefruit trees as well as grapevines. The crown gall pathogen was particularly abundant, especially on grapevines, in the areas alongside large irrigation rivers, such as the Orange and Olifants Rivers. Examples of well-developed tumours on several hosts are shown in Fig. 7 and 8.

Isolation of *Agrobacterium tumefaciens* from Diseased Plant Material

Isolation from specimens from the field

From plates of the different isolation media streaked with material from a tumour, usually 5-10 colonies showing typical *Agrobacterium* characteristics were selected. After purification, inoculation on a suitable host plant and subsequent formation of typical crown gall tumours, one or two isolates from each original tumour were selected for further study.

The isolation method with the highest success rate was isolation of the bacteria from fresh tumours making use of NA plates. The different selective media seldom supported growth during such isolations, probably due to their selectivity making them unfavourable for all agrobacteria, even those which after isolation could grow on the media.

In general, attempts to isolate the causative organism from older, dry and hard galls were unsuccessful. Usually no growth occurred on the selective media, and the NA plates were overgrown by various soil-inhabiting bacteria.

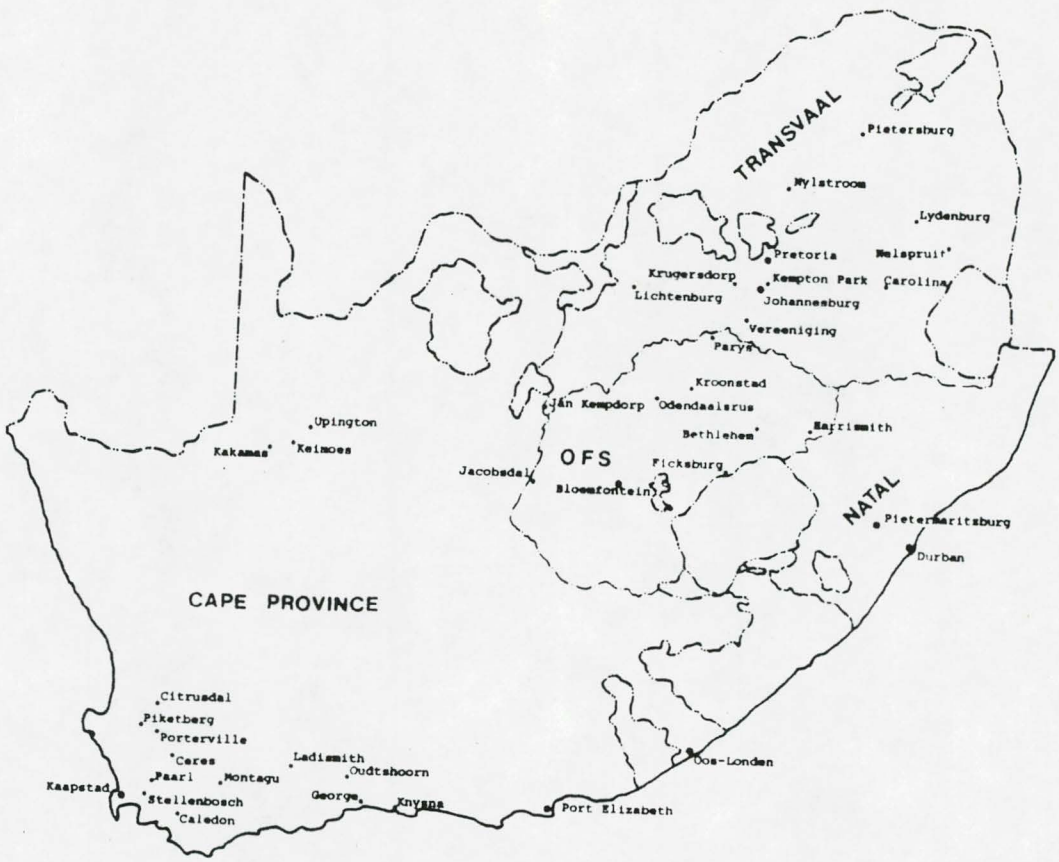


Fig. 6. Map of South Africa indicating areas where the crown gall disease has been found.

Fig. 7. Crown gall tumours on different host plants caused mainly by *A. tumefaciens* biotypes 1 and 2.

A. heavy crown gall infestation by *A. tumefaciens* on peach roots from a Stellenbosch farm.

B. A tumour on the crown area of an established young peach tree from a Paarl farm.

C. Large tumour on marguerite (*Chrysanthemum frutescens*) from a Stellenbosch nursery.

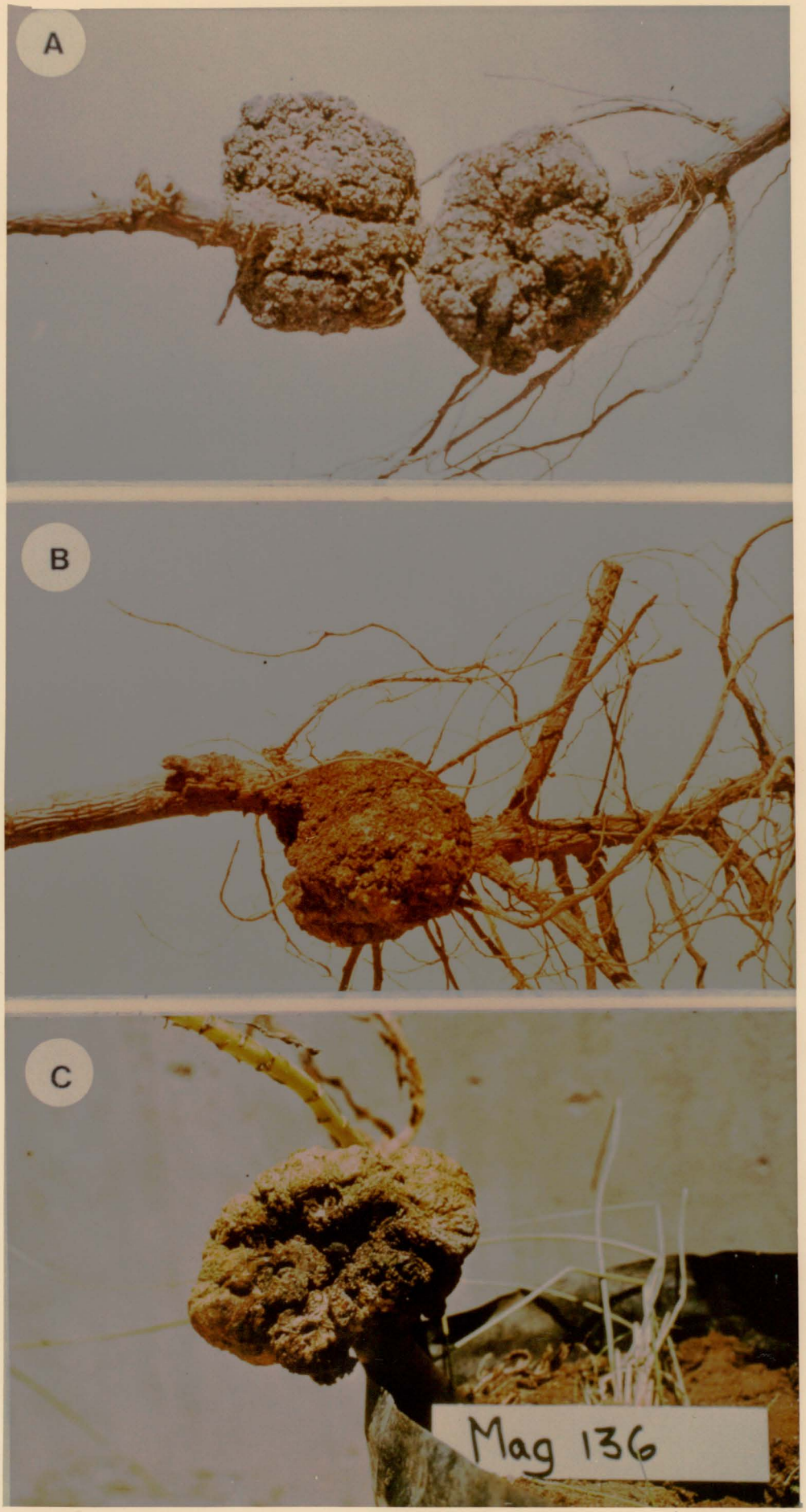


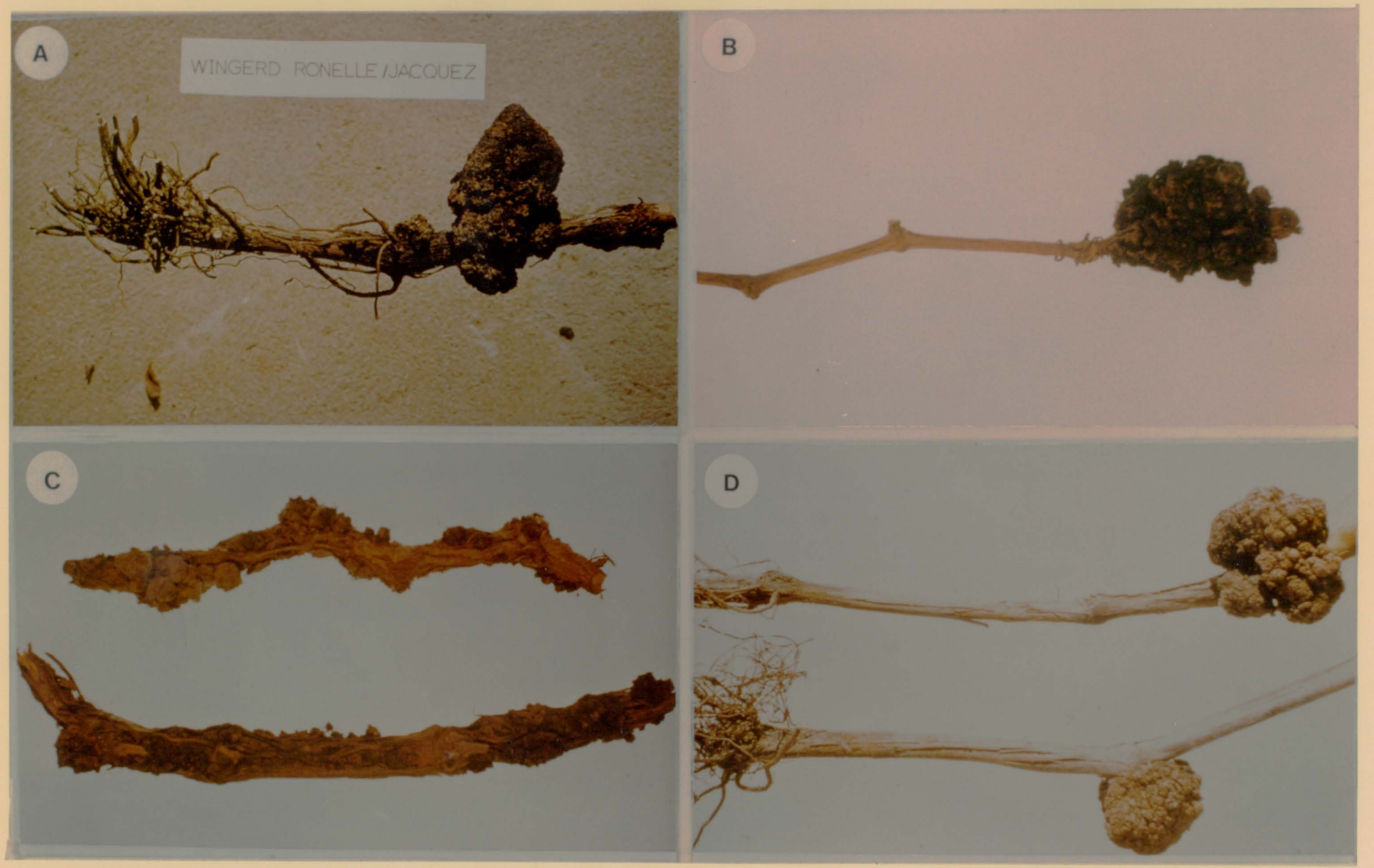
Fig. 8. Crown gall tumours on grapevine material caused mainly by *A. tumefaciens* biotype 3.

A. Crown gall developing from a wound caused by the removal of a leaf bud on Jacquez rootstock.

B. A large crown gall tumour on the graft union of Dan Ben Hannah/Jacquez nursery material.

C. Heavily infested Hanepoot (Muscat d'Alexandrie) grapevine stems from the Orange River area.

D. Large tumours on the stems of Jacquez rootstock from a Wellington nursery.



Isolation from indicator host plants

Transplantation of slices of tumours from plants in the field into stems of tobacco, datura or Jacquez seedlings (Fig. 9A,B,C,F) produced young galls which yielded very high numbers of *Agrobacterium*-like colonies. However, in a few instances crown gall symptoms did not appear on the indicator host plant stems. The pathogenic bacteria were isolated from the crown galls on the indicator plants as effectively as from fresh tumours on plants from the field.

Isolation of *Agrobacterium tumefaciens* from Soils

The recovery rate of pathogens from soils was extremely low, possibly because of the small numbers of pathogens present and the high dilutions used in this method. The best media for isolations from soils proved to be the three different selective media of Schroth *et al.* (1965) (biotype 1), New and Kerr (1971) (biotype 2) and Brisbane and Kerr (1983) (biotype 3) for the three different *Agrobacterium* biotypes. Problems were experienced with NA due to the large numbers of contaminants which grew on the plates.

Biotyping of *Agrobacterium tumefaciens* Isolates and Strains

From the biotyping results shown in Table 2, the three biotypes described by Kerr and Panagopoulos (1977) could be distinguished among the 75 *A. tumefaciens* isolates and strains tested. Thirty-nine isolates and strains belonged to biotype 1, 28 to biotype 2 and 8 to biotype 3 (Table 2). All the bacteria tested were Gram-negative motile rods which produced similar convex slightly raised, fawn-coloured, slimy, entire, circular colonies on NA plates. They produced no fluorescent pigment on King's B medium, oxidatively metabolized glucose, and showed a positive oxidase reaction.

All biotype 1 isolates and strains produced 3-ketolactose, except isolates 1887 and M27, and all except isolates 78 and 1465 (which grew at 35°C) grew at a maximum temperature of 37°C.

Fig. 9. Crown gall development on different plants.

A. Left, datura (*D. stramonium*) and right, tobacco (*N. glutinosa*) showing typical crown gall symptoms three weeks after inoculation onto 3-week-old plants.

B. Crown gall on the stem of a 3-week-old datura seedling (*D. stramonium*) 3 weeks after inoculation.

C. Crown gall on the stem of a 3-week-old tobacco seedling (*N. glutinosa*) 3 weeks after inoculation.

D. Carrot root discs inoculated with a pathogenic *A. tumefaciens* isolate 3 weeks after inoculation. The arrows point to the tumours.

E. Teratoma development on a 3-week-old tobacco seedling, 3 weeks after inoculation. Teratomas are a complex growth of highly abnormal leaves and buds in different stages of morphological development and is not similar to typical tumorous tissue as in C.

F. Tumour development of *A. tumefaciens* biotype 3 after 30 days after inoculation on the stem of a 3-week-old Jacquez seedling.

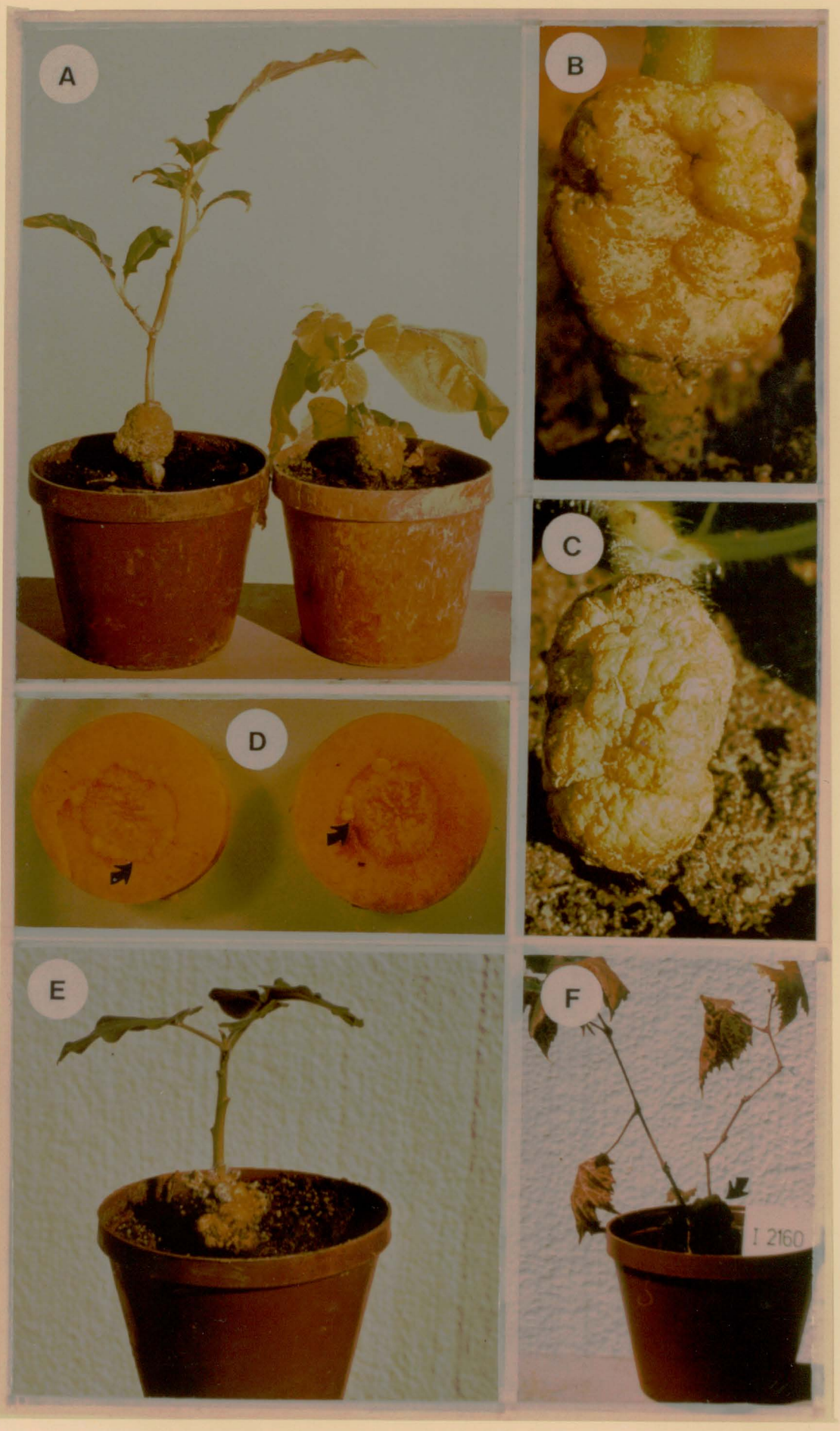


TABLE 2. Determination of biotypes among *A. tumefaciens* isolates and strains

Isolates/ strains	Test results[a] A	B	C	D	E	F
78, 1465	-	+	-	+	+	+
45d, 57, 78, 143, 172(a), 172(b), 198, 925, 1465, 1477, 1895, 2153, 2080, 2086A, 4452, B6, D286, K14, K17, K28, K21, K30, $M21_3$, $M21_4$, M37g, $M34_{11}$, $M37_{10}$, $M37_{11}$, M51g, $M51_6$, $M52_3$, $M52_5$, $M57_2$, TT9, T-37	-	+	-	+	+	+
1887, M27	-	+	-	+	+	-
39g, 39i, 39m, 39n, 41B, 46, 47, 48, 49, 70, 71, 73, A1, A5, A6, C5, I27, Z8, Z12, Z36, Z13	-	+	-	+	+	-
1671, 2077	-	+	-	+	+	-
D3, D6, D7, D8, D10	-	+	-	+	+	-
2160, 2221	-	+	-	+	+	-
2158	-	+	-	+	+	-
W7, W8	-	+	-	+	+	-
1771, 2164	-	+	-	+	+	-
305	-	+	-	+	+	-

[a]A Gram reaction.
B Motility.
C Fluorescence on King's B medium.
D Oxidase production.
E Oxidation of glucose.
F Production of 3-ketolactose.
G Growth in presence of 2% NaCl.
H Growth at 29, 35 and 37°C (the highest temperature permitting growth is indicated).
I Citrate utilization.

G	H	I	J	K		L			M	N	O
				K1	K2	L1	L2	L3			
+	35°C	-	+, alk	-	+	-	-	+	+	-	1
+	37°C	-	+, alk	-	+	-	-	+	+	-	1
+	37°C	-	+, alk	-	+	-	-	+	+	-	1
-	29°C	+	+, acid	+	-	+	+	-	-	+	2
-	29°C	-	+, acid	+	-	+	+	-	-	+	2
-	37°C	+	+, acid	+	-	-	-	-	-	+	2
-	37°C	-	+, alk	-	-	+	+	-	-	-	3
+	37°C	+	+, alk	-	-	+	+	-	-	-	3
+	37°C	-	+, alk	-	-	+	+	+	-	-	3
-	37°C	-	+, alk	-	-	+	+	+	-	-	3
+	35°C	+	+, alk	-	-	+	+	-	-	-	3

J Growth and reaction in litmus milk: +, growth;
alk, alkaline reaction;
acid, acid reaction.

K Production of acid from: K1, erythritol;
K2, melezitose.

L Utilization of: L1, malonate;
L2, tartrate;
L3, propionate.

M Growth on biotype 1 medium.

N Growth on biotype 2 medium.

O Biotype.

All produced reactions in the media containing melezitose and propionate, as can be expected of biotype 1 isolates (Kerr and Panagopoulos, 1977).

None of the biotype 1 isolates and strains showed metabolism of erythritol, malonate or tartrate. All biotype 1 isolates, except isolates K17, K30, $M37_{11}$, $M51_{6}$, $M52_{3}$, $M57_{2}$ and 4452, grew well on the selective medium of Schroth *et al.* (1965). The exceptions grew poorly on this selective medium, and strain 1887 produced hardly any growth. The biotype 1 isolates failed to grow on the biotype 2 selective medium of New and Kerr (1971).

Agrobacterium tumefaciens isolates assigned to biotype 2 grew poorly at 29°C only, except isolates D3, D6, D7, D8 and D10, which grew at 37°C. Every biotype 2 isolate, with the exception of isolates 1671 and 2077, utilized citrate. All biotype 2 isolates, with the exception of D3, D6, D7, D8 and D10, showed typical biotype 2 reactions, as described by Kerr and Panagopoulos (1977) on the media containing malonate, tartrate and propionate. Most biotype 2 isolates grew well on the selective medium of New and Kerr (1971), but isolates A5, Z12, 39g, 39i, 46, 47, 49 and 1671 grew poorly on this medium. No biotype 2 strain grew on the biotype 1 selective medium of Schroth *et al.* (1965). All biotype 2 isolates could be distinguished from the biotype 1 and 3 isolates by their acid reaction in litmus milk and acid production from erythritol.

Among the biotype 3 isolates, only W7, W8, 305 and 2158 produced a little turbidity in the 2% NaCl medium; all other biotype 3 isolates failed to grow. None of the biotype 3 isolates produced a 3-ketolactose reaction. All biotype 3 isolates grew at 37°C, except strain 305 which grew at 35 but not 37°C. Isolates 305 and 2158 were also the only biotype 3 isolates that utilized citrate as a sole carbon source. Like the biotype 1 isolates, all biotype 3 isolates produced an alkaline reaction in litmus milk. None of the biotype 3 isolates produced acid from erythritol or melezitose, and all isolates utilized malonate and tartrate. Only biotype 3 isolates W7, W8, 1771 and 2164 produced an alkaline reaction in propionate medium, whereas isolates 305, 2158, 2160 and 2221 expressed no reaction. None of the biotype 3 isolates grew

on the selective media of Schroth *et al.* (1965) for biotype 1 isolates, or New and Kerr (1971) for biotype 2 isolates.

All isolates of the three biotypes produced a clear serum zone (0.5 cm) just below the surface of the litmus milk.

Standardization of *Agrobacterium* Cell Suspensions

Colorimetric determination of absorbance of cell suspensions

The relationship between absorbance determined on the Gallenkamp CS-200 colorimeter, using blue and green filters, and the concentration of cells in suspensions of *A. tumefaciens* isolates I27 and 2080 as determined by the dilution plate count method, is shown in Table 6.

Construction of a standard graph

Separate scatterplots and regression lines of absorbance (blue and green filter readings) against dilution plate count results for each of the isolates I27 and 2080 are shown in Fig. 10 and 11.

On account of the high r^2 values for the green filter readings with both isolate I27 ($r^2 = 0.881$) and 2080 ($r^2 = 0.913$), it was decided that green filter readings would in future be used for the determination of the number of agrobacteria in a suspension by determination of the absorbance thereof. For this purpose, the results for isolates I27 and 2080 were combined for construction of the standard graph shown in Fig. 12.

Evaluation of Indicator Plants for *Agrobacterium tumefaciens* Biotype 1 and 2 Isolates

After 12 days, all replicate tobacco and datura seedlings inoculated with biotype 1 and 2 isolates showed tumour formation. Figure 9A shows the tumour symptoms caused by the

TABLE 6. Relation between absorbance of suspensions of *A. tumefaciens* isolates 2080 and I27 measured using two different filters and cell counts by a dilution plate method

Isolate	Dilution plate cell count ($X 10^9$)/ml	Colorimeter reading (absorbance)	
		Blue filter[a]	Green filter[b]
2080	5.65	0.80	0.62
	5.26	0.78	0.59
	6.87	0.88	0.66
	6.01	0.86	0.63
	3.47	0.61	0.46
	1.89	0.53	0.35
	2.77	0.60	0.42
	2.24	0.59	0.41
	1.12	0.34	0.18
	0.75	0.26	0.13
	1.63	0.32	0.16
	0.59	0.22	0.09
I27	8.75	-	0.67
	7.80	-	0.68
	7.41	-	0.64
	7.01	-	0.63
	5.46	0.62	0.38
	4.66	0.63	0.44
	4.20	0.58	0.37
	3.20	0.65	0.42
	2.31	0.41	0.24
	1.48	0.47	0.34
	1.25	0.39	0.19
	1.07	0.42	0.27

[a]Maximum transmittance at 470 nm.
[b]Maximum transmittance at 540 nm.

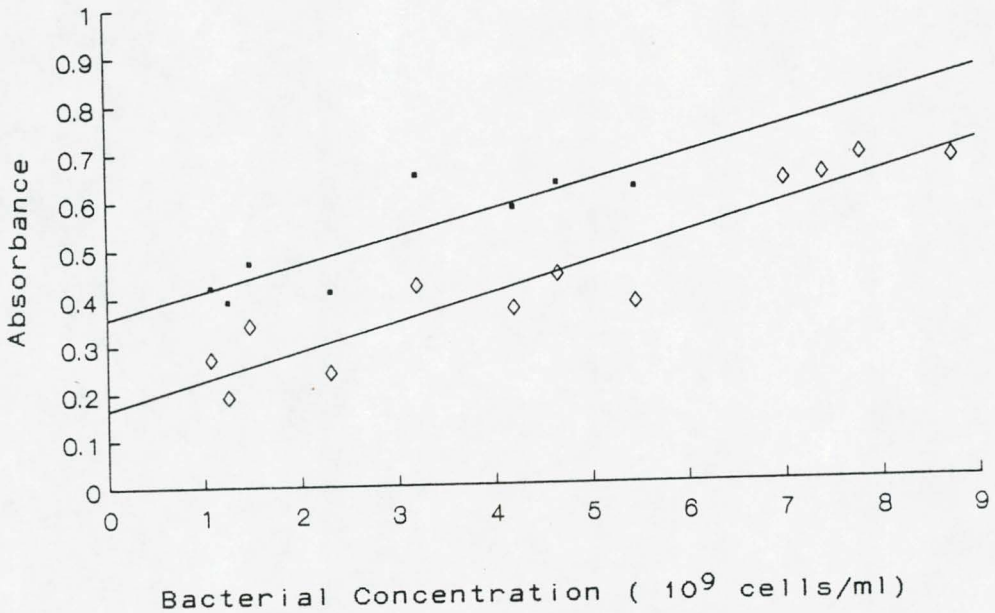


Fig. 10. Scatterplots and regression lines of absorbance with two different filters against dilution plate count of *A. tumefaciens* isolate I27.

▪ = blue filter [$y = 0.357 + 0.055(\pm 0.014)x$, $r^2 = 0.724$];
◇ = green filter [$y = 0.166 + 0.059(\pm 0.007)x$, $r^2 = 0.881$]

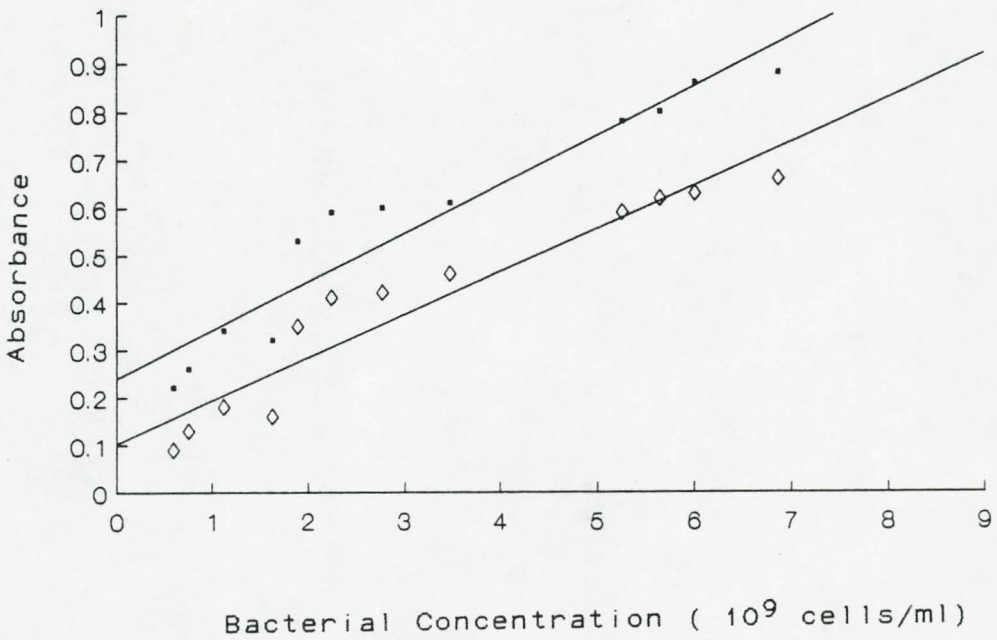


Fig. 11. Scatterplots and regression lines of absorbance with two different filters against dilution plate count of *A. tumefaciens* isolate 2080

▪ = blue filter [$y = 0.241 + 0.102(\pm 0.009)x$, $r^2 = 0.916$];
◇ = green filter [$y = 0.104 - 0.091(\pm 0.009)x$, $r^2 = 0.913$]

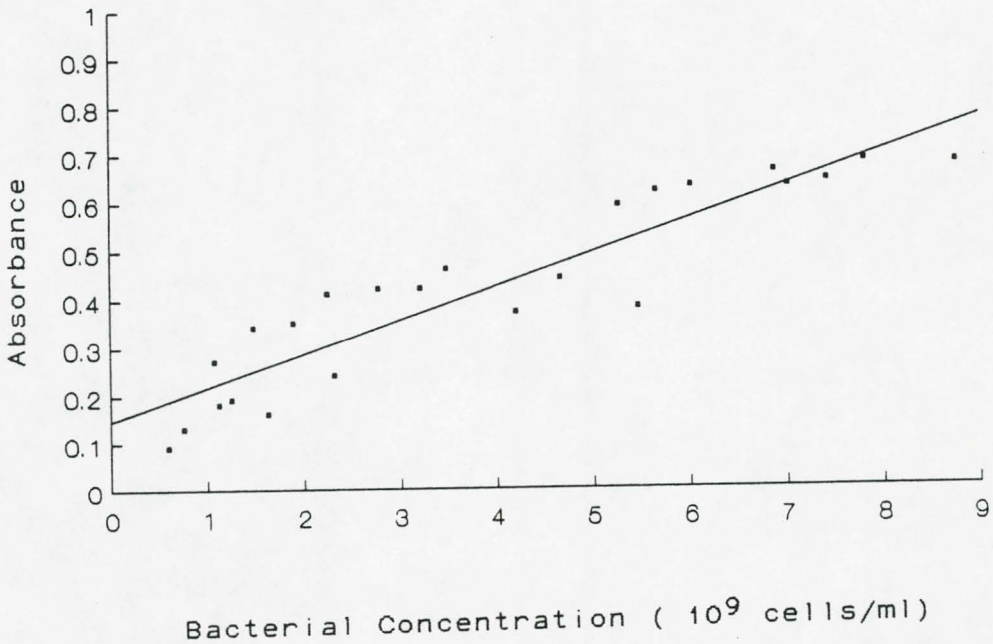


Fig. 12. Combined scatterplot and regression line of absorbance with green filter against dilution plate count of *A. tumefaciens* isolates I27 and 2080. The regression line [$y = 0.147 + 0.069(\pm 0.006)x$, $r^2 = 0.849$] has been used as a standard graph for the routine determination of *Agrobacterium* cell concentrations from absorbance readings.

pathogenic biotype 1 isolate $M34_{11}$ on tobacco and datura 3 weeks after inoculation and Fig. 9B and C those caused by biotype 2 isolate 2077. The tumours produced on datura and tobacco were typically large unorganized masses of cells, and light in colour. Both plant species were sturdy and easy to cultivate and manipulate.

Broadbean and tomato plants showed tumour formation only after 22 days. Developed tumours were approximately three to six times smaller than those produced on tobacco and datura. Not all replicate broadbean and tomato plants produced tumours from inoculation by pathogenic isolates, for example, 42 days after inoculation of 12 plants with each pathogen, only 10, 11, eight (two died), eight (one died) and 10 broadbean plants produced tumours following inoculation with biotype 1 isolates $M21_3$, M27, $M37_{11}$, and biotype 2 isolates 1671 and 2077, respectively. With tomato similar results were obtained, in that only nine (two died), 10, seven (one died), 10 and eight (one died) plants, respectively, produced tumours from inoculation with these isolates. Death of some tomato and broadbean plants during the experimentation, as a result of secondary infection by either bacteria or fungi was not unusual. The broadbean was especially sensitive to a black discolouration around the wounded areas, probably caused by bacteria, whereafter these plants died very quickly. Broadbean and tomato plants were also quite difficult to cultivate and relatively low germination percentages (62 and 51%, respectively) were obtained.

The sunflower seedlings showed good tumour development on most plants 8 or 10 days after inoculation with the biotype 1 and 2 test isolates of *A. tumefaciens* (Table 3). All surviving inoculated plants showed tumour development. The plants were easy to cultivate and easily manageable, although they tend to become quite large after a while.

The carrot discs produced tumours (Fig. 9D) only after 16 days, when some of the discs were spoiled and appeared to be contaminated by soft-rotting bacteria and certain unknown fungi. Three carrot root discs inoculated with the pathogenic *A. tumefaciens* biotype 1 isolates M27 and $M37_{11}$ and biotype 2 isolate 1671 failed to produce tumours.

TABLE 3. Tumour development on sunflower seedlings inoculated with *A. tumefaciens* biotype 1 and 2 isolates

Biotype	Isolate	Days after inoculation	Plants with tumours[a]
1	2086A	10	5
1	$M34_{11}$	10	5
1	1887^{11}	10	5
1	172(a)	8	5
2	2077	8	5
2	39g	10	5
2	46	10	5
2	C5	10	4[b]
-	Control	10	0

[a]Numbers of plants with crown gall tumours out of a total of five inoculated.
[b]One plant dead.

Evaluation of Indicator Plants for *Agrobacterium tumefaciens* Biotype 3 Isolates

Vegetatively propagated rootstock of *Vitis* species

Of the rootstock cultivars tested, Jacquez, Sultana and Hanepoot (Muscat d'Alexandrie) appeared to be the most susceptible to infection by *A. tumefaciens* biotype 3 isolate 2221, as indicated by the formation of tumours (Table 4). On the Jacquez and Sultana material, eight and nine, respectively, of the 10 test plants produced tumours, only 3 weeks after inoculation. Seven Hanepoot, six Richter 99, four 143B, four Colombard and one Teleki, of 10 plants of each inoculated, produced tumours by 4 weeks after inoculation. No other plants formed tumours after 4 weeks. No tumours were produced on either the Ramsey or 101-14 cultivars. The tumours were all well developed typical tumorous tissue which was clearly distinguishable from the callous tissue of the sterile water controls. Tumours on Sultana, with diameters from 3.0 to 4.5 cm, were much bigger than those produced on the other cultivars, where the diameters ranged from 1.0 to 2.5 cm.

Propagation of rootstock material for the purpose of host plant testing was very time consuming. As Jacquez, which is probably the most common rootstock cultivar, was one of the most susceptible cultivars for *A. tumefaciens* isolate 2221, it was decided to propagate only Jacquez seedlings for further *A. tumefaciens* pathogenicity tests.

Vitis cv. Jacquez (*Vitis aestivalis* x *Vitis cinerea* x *Vitis vinifera*) seedlings

All the Jacquez seedlings showed tumour formation within 12 days after inoculation with all the *A. tumefaciens* biotype 3 isolates, except isolate W8 (Table 5). Tumours were well developed after 30 days. After 40 days tumour sizes ranged from 0.4 to 2.3 cm in diameter. The largest tumours after 40 days ranged from 2.2 to 2.4 cm in diameter and were caused by isolate 2221. Figure 9F shows the tumour formation caused by biotype 3 isolate 2160 on Jacquez seedlings.

TABLE 4. Tumour development on different *Vitis* rootstock cultivars inoculated with *A. tumefaciens* biotype 3 isolate 2221

Vitis	Plants[a] showing tumours after Weeks				
	2	3	4	5	6
143B Mgt[b]	0	2	4	4	4
Teleki[c]	0	1	1	1	1
Jacquez[d]	7	8	9	9	9
Ramsey[e]	0	0	0	0	0
99 Richter[f]	0	4	6	6	6
Hanepoot[g]	2	6	7	7	7
Colombar[h]	2	4	4	4	4
Sultana[i]	8	9	9	9	9
101-14 Mgt[j]	0	0	0	0	0

[a]Plants showing crown gall tumours out of a total of 10 inoculated.
[b]*Vitis vinifera* cv. *aramon* x *Vitis riparia*.
[c]*Vitis berlandieri* x *Vitis riparia*.
[d]*Vitis aestivalis* x *Vitis cinerea* x *Vitis vinifera*.
[e]*Vitis champini*.
[f]*Vitis berlandieri* var. Las Sorres x *Vitis rupestris*.
[g]*Vitis vinifera*.
[h]*Vitis vinifera*.
[i]*Vitis vinifera*.
[j]*Vitis riparia* x *Vitis rupestris*.

TABLE 5. Tumour development on seedlings of *Vitis* cv. Jacquez (*V. aestivalis* x *V. cinerea* x *V. vinifera*) inoculated with different *A. tumefaciens* biotype 3 isolates

Isolate/ strain	Plants with tumours[a] (mean per replication)	Mean tumour diameter[b] (cm)
2221	2	2.30
305	2	2.00
2158	2	1.03
W7	2	1.00
2164	2	0.80
1771	2	0.57
2160	2	0.40
W8	0	0.00
control	0	0.00

[a]Two plants were inoculated with each isolate/strain in each of three replications. All susceptible plants produced visually detectable tumours 12 days after inoculation. Observations were however continued for 8 weeks.
[b]Mean diameter of all tumours produced in the three replications 40 days after inoculation.

Tobacco (*Nicotiana glutinosa*), datura (*Datura stramonium*) and sunflower (*Helianthus annuus*) seedlings

All seven *A. tumefaciens* biotype 3 isolates and strains which were tested produced tumours on tobacco within 3 weeks. However, on datura, only isolates 1771 and W7 produced tumours; no tumours were produced by isolates W8, 305, 2160, 2164 and 2221 up to 8 weeks after inoculation. No sunflower seedlings (*H. annuus* var. *nanus*) showed tumour formation with any of the biotype 3 isolates, even after a period of 6 weeks after inoculation. The experiment was repeated three times, also with the sunflower variety *H. annuus* var. *grandiflorus*, but the same negative results were obtained.

Serological Studies

Evaluation of media for production of *Agrobacterium tumefaciens* antigens

The *A. tumefaciens* strains used as antigens for the production of antisera grew well on both the YMA and NA plates. It was visually apparent, however, that much more extracellular slime was produced during growth on YMA than on NA plates. Isolates TT9 (biotype 1) and 2221 (biotype 3) grew relatively well on PGYA with little slime production, but isolate 2077 (biotype 2) grew poorly, if at all. The biggest cell yields with the least amount of slime production were obtained on NA plates.

Cells collected from two NA plates, after two centrifugations and resuspension in 20 ml distilled water, needed 85 ml sterile distilled water (mean of results for three isolates replicated three times) for dilution to an absorbance corresponding to 5×10^9 cells/ml; YMA-grown cells required 32 ml, and PGYA-grown cells 6 ml. The extracellular polysaccharide slime layer surrounding *A. tumefaciens* strains was easily removed by the centrifugation, as was also found by Cagle (1975).

Initial trials of serological test techniques and specificities of antisera

Reactions in the Wassermann tube agglutination tests were best when the test tubes were incubated in a waterbath at 47°C for 4-5 h then kept overnight at 4°C before the final O-reactions were recorded. Distinct differences were observed in the agglutination reactions of the type A and B antigens in that higher O-agglutination titres and flagellar (H) reactions were shown by the type A antigens (Tables 7 and 8). No flagellar reactions were observed with the Type B preparations. Also, no flagellar reactions were observed with heated antigens. In respect of the O-reactions, no differences were observed between unheated and the corresponding heated antigens. The procedure chosen for the determination of O- and H-agglutination reactions of the *A. tumefaciens* isolates thus used unheated antigen preparations

TABLE 7. Tube agglutination and Ouchterlony immunodiffusion tests with three antisera against *Agrobacterium* Type A preparations (minimally washed) of a biotype 1 (TT9) and two biotype 2 (D3, 2077) isolates

Antigen[a]	O-agglutination titre with antiserum			H-agglutination titre with antiserum			Ouchterlony precipitin bands with antiserum		
	TT9	D3	2077	TT9	D3	2077	TT9	D3	2077
TT9	2560	-[b]	-	320	-	-	+(I)[c]	-	-
D3	-	5120	160	-	160	-	-	+(I)	+(PI)[d]
2077	-	320	2560	-	-	320	-	+(PI)	+(I)

[a]Type A preparations, without further modification for the agglutination tests but treated with phenol for the Ouchterlony immunodiffusion tests.
[b]No agglutination or precipitin band formation.
[c]Identity of diffusible antigen(s) indicated by complete fusion of precipitin bands.
[d]Partial identity of diffusible antigen(s) indicated by fusion of precipitin bands with spur formation.

TABLE 8. Tube agglutination and Ouchterlony immunodiffusion tests with three antisera against *Agrobacterium* Type B preparations (well washed) of a biotype 1 (TT9) and two biotype 2 (D3, 2077) isolates

Antigen[a]	O-agglutination titre with antiserum			Ouchterlony precipitin bands with antiserum		
	TT9	D3	2077	TT9	D3	2077
TT9	2560	-[b]	-	+(I)[c]	-	-
D3	40	2560	80	-	+(I)	-
2077	-	-	640	-	-	+(I)

[a]Type B preparation, without further modification for the agglutination tests but treated with phenol for the Ouchterlony immunodiffusion tests.
[b]No agglutination or precipitin band formation.
[c]Identity of diffusible antigen(s) indicated by complete fusion of precipitin bands.

and incubation of the reaction mixtures at 47°C for 4-5 h, followed by overnight incubation at 4°C.

The best results in the Ouchterlony immunodiffusion tests with both the type A and B cell preparations were obtained with phenol-treated cells. The phenol-treated preparations provided the best antigens which produced thin and clearly defined precipitin bands in their reaction to homologous antiserum. The heat-treated cell preparations also provided thin and clearly defined precipitin bands, although not as sharp as those of the phenol-treated cells. Untreated and ultrasonically disintegrated type A and B cell preparations, as well as the liquid supernatant from centrifuged nutrient broth cultures of the test isolates produced very weak and diffuse precipitin bands. Heat-treated antigens and, with one exception (isolate 2077 versus antiserum 2077), phenol-treated antigens produced patterns of precipitin bands similar to those produced by the corresponding untreated antigens (Types A and B). On the basis of these results in the initial trials, phenol-treated cell preparations were used in further Ouchterlony tests.

In the cross agglutination tests with three type A antigen preparations (Table 7) it appeared as if *A. tumefaciens* isolate TT9 had no serological O-antigenic component in common with *A. tumefaciens* isolates D3 and 2077. *Agrobacterium tumefaciens* isolates D3 and 2077 had a minor O-antigenic component in common; however, they also had strain-specific O-antigens that did not cross react, so that homologeous titres were much higher than titres resulting from the common O-antigenic component. The three isolates also had strain-specific H-antigens which did not cross react. The relationships shown by the cross agglutination tests were supported by the results of the Ouchterlony cross immunodiffusion tests with phenol-treated Type A antigen preparations (Table 7).

In corresponding cross agglutination tests with the type B preparations (Table 8) it was again apparent that *A. tumefaciens* isolates TT9, D3 and 2077 had their own strain-specific O-antigenic components. *Agrobacterium tumefaciens* isolate D3 also had minor O-antigenic components that reacted with the antisera of *A. tumefaciens* isolates TT9 and 2077. However,

these apparently common O-antigenic components did not show reactions in the tests with isolates TT9 and 2077 versus the D3 antiserum. The relatioships indicated by the cross agglutination tests were not completely supported by the Ouchterlony cross immunodiffusion tests with phenol-treated Type B antigens (Table 8), as no major or minor cross reactions were observed.

In cross agglutination tests with seven different type B cell preparations and their antisera (Table 9), it was clear that each isolate had strain-specific O-antigens that did not cross react. However, some strains seemed to share minor O-antigenic components, according to single but not cross agglutination reactions. The reactions of *A. tumefaciens* isolate D3 with the TT9 and 2077 antisera have been noted; other reactions were those of *A. tumefaciens* isolate 2080 with isolate 2221 antiserum, isolate 2077 with isolate 305 antiserum and isolate 2221 with the antisera of isolates $M34_{11}$, D3 and 305. Usually these reactions were at much lower titres than the reactions of the isolates with their homologous antisera, but the reactions of isolates 2077 and 2221 with antiserum 305 exhibited titres almost as high and as high, respectively, as the titres of their homologous reactions.

Both type A and type B preparations displayed enough antigenic specificity for the grouping of the *A. tumefaciens* isolates according to their agglutination reactions with the antisera. The O-reactions of the minimally washed type A preparations were based on more superficicial O-antigenic components than those of the well-washed type B preparations. The two groups of antigenic components were designated surface O-antigens (0/S) and deep O-antigens (O/D), respectively.

Wassermann tube agglutination tests

Results of tube agglutination studies with type A and B cell preparations of the *A. tumefaciens* isolates and antisera against selected type A and B cell preparations are shown in Tables 10 and 11.

TABLE 9. Tube agglutination tests with seven antisera against *Agrobacterium* Type B preparations (well washed) of three biotype 1 (2080, $M34_{11}$, TT9), two biotype 2 (D3, 2077) and two biotype 3 (2221, 305) isolates

Antigen (Type B preparation)	O agglutination titre with antiserum against						
	Biotype 1			Biotype 2		Biotype 3	
	2080	$M34_{11}$	TT9	D3	2077	2221	305
2080	1280	-[a]	-	-	-	40	-
$M34_{11}$	-	1280	-	-	-	-	-
TT9	-	-	2560	-	-	-	-
D3	-	-	40	2560	80	-	-
2077	-	-	-	-	640	-	320
2221	-	40	-	40	-	1280	1280
305	-	-	-	-	-	-	1280

[a]No agglutination.

TABLE 10. Serological grouping of *Agrobacterium* isolates and strains according to agglutination reactions based on surface O- (O/S) and H-antigens

Isolate/ strain (antigen)	O-agglutination with antiserum			O-sero= group		H-agglutination with antiserum			H-sero= group	
	TT9	D3	2077	O/S	SG[a]	TT9	D3	2077	H	SG[b]
TT9	2560[c]	-[d]	-	I	a	320	-	-	I	a
2086A	1280	-	-		b	320	-	-	I	a
2080	640	-	-		c	160	-	-	I	b
198	640	-	-		c	320	-	-	I	a
T-37	640	-	-		c	160	-	-	I	b
4452	640	-	-		c	-	-	-	-	-
B6	640	-	-		c	-	-	-	-	-
D286	320	-	-		d	-	-	-	-	-
W7	160	-	-		e	-	-	-	-	-
W8	160	-	-		e	-	-	-	-	-
$M52_3$	160	-	-		e	-	-	-	-	-
M51g	80	-	-		f	-	-	-	-	-
$M51_6$	80	-	-		f	-	-	-	-	-
1465	640	320	-	II	a	160	40	-	II	a
925	320	160	-		b	-	-	-	-	-
$M52_5$	320	80	-		c	80	-	-	I	c
M129	80	80	-		d	-	-	-	-	-
$M37_6$	160	-	320	III	a	-	-	-	-	-
M37g	160	-	160		b	-	-	-	-	-
$M37_{11}$	160	-	80		c	-	-	-	-	-
$M21_3$	80	-	160		d	-	-	-	-	-
2153	80	-	160		d	-	-	-	-	-
$M37_{10}$	80	-	80		e	-	-	-	-	-
$M21_4$	80	-	80		e	-	-	-	-	-
D10	320	640	80	IV	a	-	-	-	-	-
1771	-	80	-	V	a	160	-	-	I	d
2160	-	80	-		a	-	-	-	-	-
2221	-	40	-		b	-	-	-	-	-
D7	-	5120	640	VI	a	-	-	-	-	-
D3	-	5120	160		b	-	-	-	-	-
D6	-	2560	80		c	-	-	-	-	-
71	-	1280	320		d	-	40	-	II	a
D8	-	1280	160		e	-	-	-	-	-
41B	-	320	2560		f	-	-	320	III	a
2077	-	320	2560		f	-	-	320	III	a
K84	-	320	320		g	-	-	-	-	-
A6	-	160	5120		h	-	40	320	IV	a
C5	-	80	5120		i	-	-	160	III	b
39m	-	80	5120		i	-	-	320	III	a
39n	-	80	2560		j	-	-	20	III	d
73	-	80	2560		j	-	-	160	III	b
A1	-	80	640		k	-	-	80	III	c
39i	-	80	160		l	-	-	-	-	-
305	-	80	80		m	-	-	-	-	-
Z12	-	40	2560		n	-	-	320	III	a
Z36	-	40	2560		n	-	-	320	III	a
I27	-	40	640		o	-	-	-	-	-
A5	-	40	640		o	-	-	-	-	-

TABLE 10. /continued/

Isolate/ strain (antigen)	O-agglutination with antiserum			O-sero= group		H-agglutination with antiserum			H-sero= group	
	TT9	D3	2077	O/S	SG[a]	TT9	D3	2077	H	SG[b]
48	-	-	2560	VII	a	-	-	320	III	a
Z8	-	-	640		b	-	-	20	III	d
46	-	-	640		b	-	-	160	III	b
49	-	-	640		b	-	-	320	III	a
47	-	-	640		b	-	-	320	III	a
70	-	-	320		c	-	-	-	-	-
39g	-	-	320		c	-	-	-	-	-
143	-	-	160		d	-	-	-	-	-
45d,57,78, 172(a), 172(b), 1477,1671, 1887,1895, 2158,2164, K14,K17, K21,K28, K30,$M34_{11}$, M52g,M57g, Z13	No reactions detected					No reactions detected				

[a]Subgroup of O/S main group.
[b]Subgroup of H main group.
[c]Titre.
[d]No reaction.

TABLE 11. Serological grouping of *Agrobacterium* isolates and strains according to agglutination based on 'deep' O-antigens (O/D)

Isolate/ strain (antigen)	O-agglutination with antiserum							O-serogroup	
	TT9	D3	2077	$M34_{11}$	2080	2221	305	O/D-Group	Sub-Group
TT9	1280[a]	-[b]	-	-	-	-	-	I	a
T-37	640	-	-	-	-	-	-		b
1465	640	-	-	-	-	-	-		b
D286	320	-	-	-	-	-	-		c
M129	40	-	-	-	-	-	-		d
Z12	40	-	-	-	-	-	-		d
M51g	160	-	-	-	80	-	80	II	
D10	640	-	40	-	-	-	-	III	
925	640	160	-	-	-	-	-	IV	a
71	320	160	-	-	-	-	-		b
1895	160	160	-	-	-	-	-		c
Z8	80	20	-	-	-	-	-		d
78	40	80	-	-	-	-	-		e
1771	640	320	-	-	-	-	320	V	a
70	80	160	-	-	-	-	40		b
$M52_5$	320	80	-	40	80	40	320	VI	
39n	80	160	640	-	-	-	640	VII	a
A1	20	80	640	-	-	-	160		b
D3	40	2560	80	-	-	-	-	VIII	
D7	320	640	320	-	-	40	-	IX	a
D8	320	640	160	-	-	40	-		b
D6	-	2560	80	-	-	-	320	X	a
41B	-	320	640	-	-	-	160		b
C5	-	160	640	-	-	-	320		c
39m	-	80	640	-	-	-	640		d
A5	-	80	320	-	-	-	640		e
K84	-	80	320	-	-	-	640		e
A6	-	40	640	-	-	-	160		f
Z36	-	160	640	-	-	-	-	XI	
172(a)	-	160	-	160	-	-	-	XII	a
172(b)	-	160	-	160	-	-	-		a
2221	-	40	-	40	-	1280	1280	XIII	a
2164	-	40	-	40	-	1280	1280		a
W7	-	40	-	40	-	640	640		b
2160	-	40	-	40	-	160	320		c
49	-	-	640	-	-	-	-	XIV	a
48	-	-	640	-	-	-	-		a
46	-	-	640	-	-	-	-		a
47	-	-	320	-	-	-	-		b
73	-	-	320	-	-	-	-		b
I27	-	-	320	-	-	-	-		b
39g	-	-	80	-	-	-	-		c
2077	-	-	640	-	-	-	80	XV	a

TABLE 11. /continued/

Isolate/ strain (antigen)	O-agglutination with antiserum							O-serogroup	
	TT9	D3	2077	$M34_{11}$	2080	2221	305	O/D-Group	Sub-Group
$M34_{11}$	-	-	-	1280	-	-	-	XVI	a
K14	-	-	-	1280	-	-	-		a
K21	-	-	-	1280	-	-	-		a
K17	-	-	-	640	-	-	-		b
K28	-	-	-	640	-	-	-		b
K30	-	-	-	640	-	-	-		b
$M51_6$	-	-	-	640	-	-	-		b
57	-	-	-	320	-	-	-		c
1477	-	-	-	80	-	-	-		d
198	-	-	-	20	-	-	-		e
2080	-	-	-	-	1280	40	-	XVII	a
2086A	-	-	-	-	640	160	-		b
305	-	-	-	-	-	-	1280	XVIII	a
$M52_3$	-	-	-	-	-	-	160		b
39i,45d, 143,1671, 1887,2153, 2158,4452, B6,$M21_3$, $M21_4$,M37g, $M37_6$,$M37_{10}$, $M37_{11}$,$M52_g$, $M57_2$,W8	No reactions detected								

[a]Titre.
[b]No reaction.

A preliminary classification of the isolates in serogroups based on the presence or absence of agglutination of type A (minimally washed) cells with three antisera against type A cells showed seven main surface O-antigenic groups (O/S groups), with groups O/S-I and O/S-VI being the largest groups (Table 10). Subgroups were also defined on the basis of agglutination titre differences. Group O/S-VI contained the largest number of subgroups (15) followed by group O/S-I with six subgroups. Group O/S-IV contained only a single isolate. Flagellar (H-) reactions were shown by 24 isolates (Table 10). Some isolates of serogroups O/S-I, O/S-II and O/S-V were of the same H-reaction group, namely group H-I. Most isolates of groups O/S-VI and VII belonged to the H-III group. Single isolates of groups O/S-II and VI belonged to the H-II and III groups. However, many isolates showed no H-reaction and could therefore not be grouped on the basis of flagellar antigens. Such isolates either had no flagellar antigens, or had flagellar antigens which did not react with the three test antisera.

In a serogrouping based on the reactions of type B cells (well washed), 18 serogroups were distinguished (Table 11). This serogrouping made use of the deeper O-antigenic (O/D) components. More serogroups were defined than when unwashed cells were used, partly because more antisera were used (seven compared to three in the case of the unwashed cells). The largest serogroup was O/D-XVI containing five subgroups distinguished by titre differences. No H-reactions (flagellar reactions) were observed with the O/D preparations as the flagella would have been removed by the washing.

The fewer O/S groups than O/D groups indicate that the study of the deeper O-antigens gave a finer classification than the study of the surface O-antigens alone. In a later section (Table 14) the two classifications are combined to provide a single seroclassification of the isolates based on their deduced antigenic structures. Tables 10 and 11 both list *A. tumefaciens* isolates that produced no reaction with any of the antisera. These isolates were classified as unknown serotypes.

Ouchterlony immunodiffusion tests

Precipitin bands produced by phenol-treated type A (minimally washed) preparations of various isolates with antiserum against TT9 type A cells are shown in Fig. 13A,C,D,F and 14. The homologous isolate TT9 and heterologous isolate T-37 produced precipitin bands showing complete identity. Precipitin bands of partial identity with that of isolate TT9 were formed by the heterologous antigens B6, D10, $M21_3$, $M21_4$, $M37_{10}$, $M37_{11}$, M37g, M51g, $M52_5$, M129, 198, 925, 1465, 2153, 2080, 2086A and 4452 with antiserum TT9. The precipitin bands of both the homologous and heterologous isolates tended to be close to the antigen wells (Fig. 14) and the homologous precipitin bands were thicker than the heterologous precipitin bands. Isolate $M52_3$ produced two thin precipitin bands with the TT9 antiserum.

Precipitin reactions of the homologous and different heterologous isolates with the antiserum against type A cells of isolate D3 are illustrated in Fig. 13E and 15. Isolates D6, D7, D8 and D10 produced precipitin bands of identity with that of isolate D3. Isolates A1, A6, C5, $M52_5$, M129, 39m, 39n, 41B, 71, 925, 1465, 1771 and 2077 produced precipitin bands of partial identity to the homologous D3 band. The homologous precipitin band was thicker than the heterologous bands. The non-pathogenic agrocin-producing strain K84 reacted with the D3 antiserum, producing a band of partial identity with the D3 band.

With antiserum against type A cells of isolate 2077, bands of identity with that of isolate 2077 were formed by isolates A1, A6, C5, Z8, Z12, 41B, 46, 48, and 49 (Fig. 16 and 17). Precipitin bands of partial identity with that of 2077 were produced by isolates A5, D3, D6, D7, D8, D10, I27, $M21_3$, $M21_4$, M37g, $M37_6$, $M37_{10}$, $M37_{11}$, $M57_2$, Z36, 39g, 39i, 39m, 39n, 70, 71, 73, 143 and 2153. The non-pathogenic agrocin-producing strain K84 also produced a band of partial identity with that of isolate 2077.

Type A (minimally washed) or type B (well washed) cell preparations of the biotype 3 isolates W7, W8, 305, 1771, 2160, 2164 and 2221 displayed no homologous or heterologous precipitin reactions with antisera against type B cells of isolates 305 and 2221. However, on closer

Fig.13. Ouchterlony immunodiffusion precipitin reactions of various phenol-treated *A. tumefaciens* cell preparations (antigen) with antisera against TT9 type A, $M34_{11}$ type B and D3 type A cells.

A. TT9 antiserum (central wells) versus type A antigen of isolates:

a. TT9 (1), 1477 (2), TT9 (3), D286 (4), TT9 (5) and 78 (6).

b. TT9 (1), T-37 (2), TT9 (3), 172(a) (4), TT9 (5) and $M52_5$ (6).

c. TT9 (1), K30 (2), TT9 (3), Z12 (4), TT9 (5) and 305 (6).

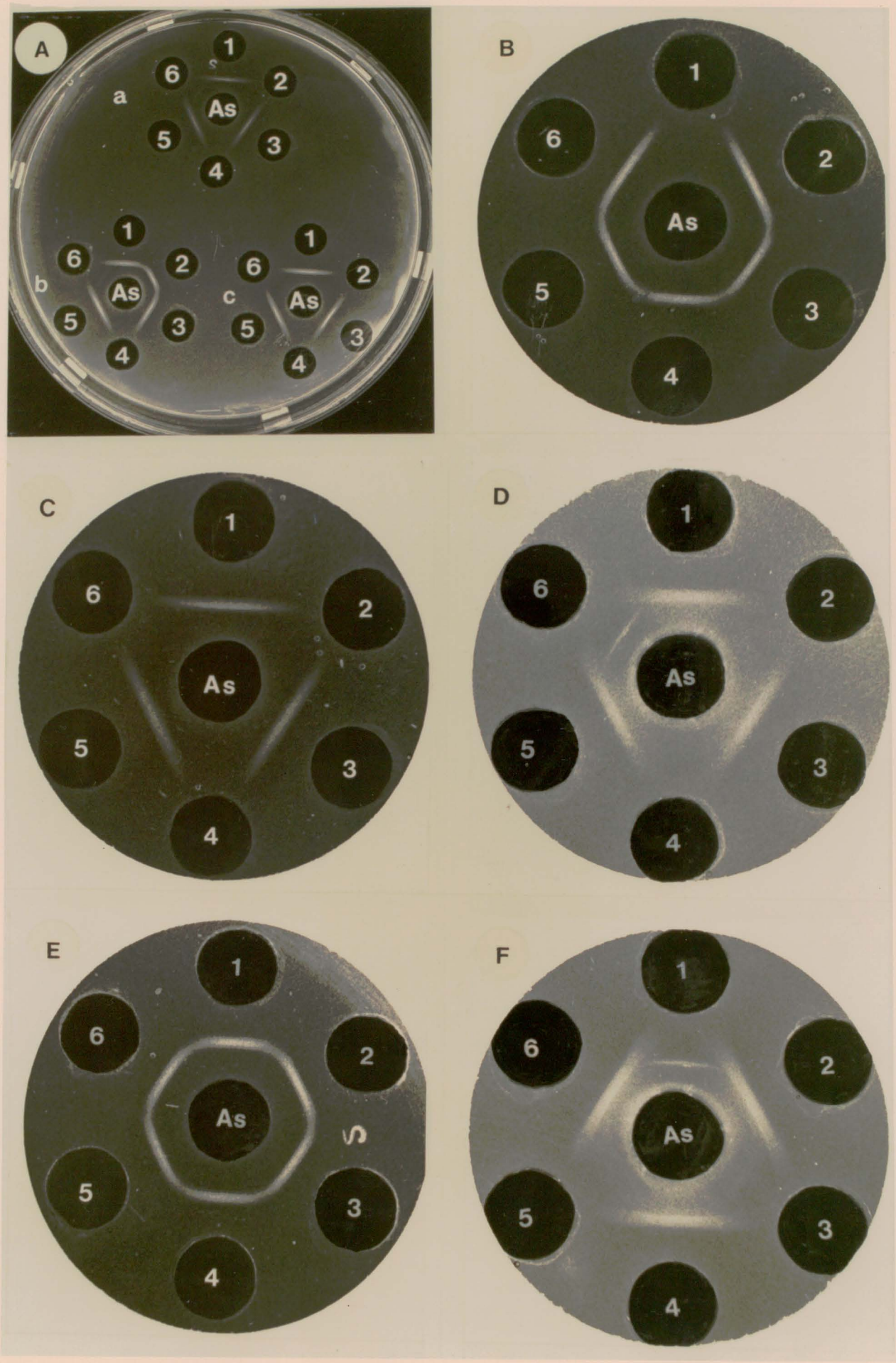
B. $M34_{11}$ antiserum (central well) versus type B antigen of isolates 925 (1), $M34_{11}$ (2), 1477 (3), $M34_{11}$ (4), $M51_6$ (5), and $M34_{11}$ (6).

C. TT9 antiserum (central well) versus type A antigen of isolates TT9 (1), 57 (2), TT9 (3), 39n (4), TT9 (5) and C5 (6).

D. TT9 antiserum (central well) versus type A antigen of isolates TT9 (1), D286 (2), TT9 (3), 1477 (4), TT9 (5) and 1895 (6).

E. D3 antiserum (central well) versus type A antigen of isolate D3 in all peripheral wells.

F. TT9 antiserum (central well) versus type A antigen of isolates 1465 (1), TT9 (2), 71 (3), TT9 (4), 39n (5) and TT9 (6).



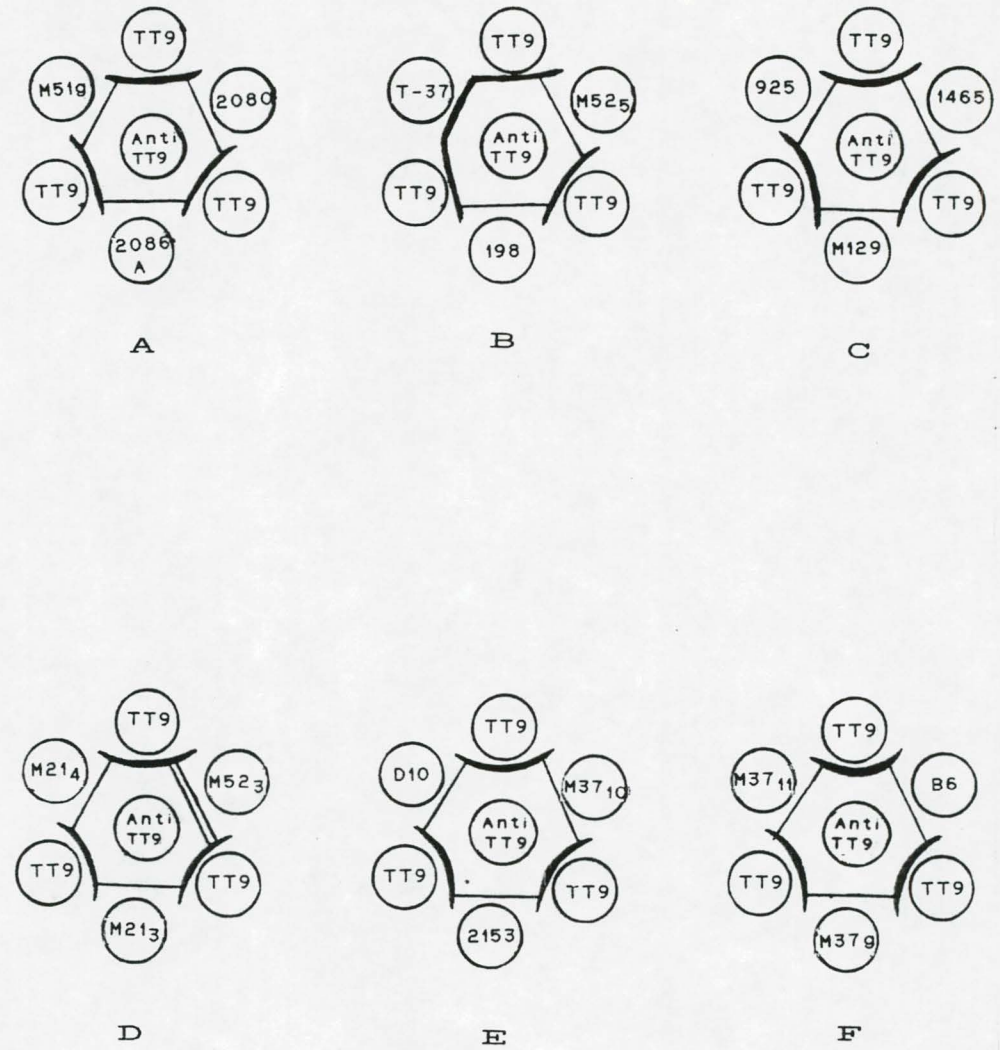


Fig. 14. Ouchterlony immunodiffusion precipitin reactions of type A (minimally washed) cell preparations of *A. tumefaciens* isolates with antiserum against type A cells of isolate TT9. The reaction of isolate 4452 is not shown, but it was similar to that of isolate $M21_4$ (D).

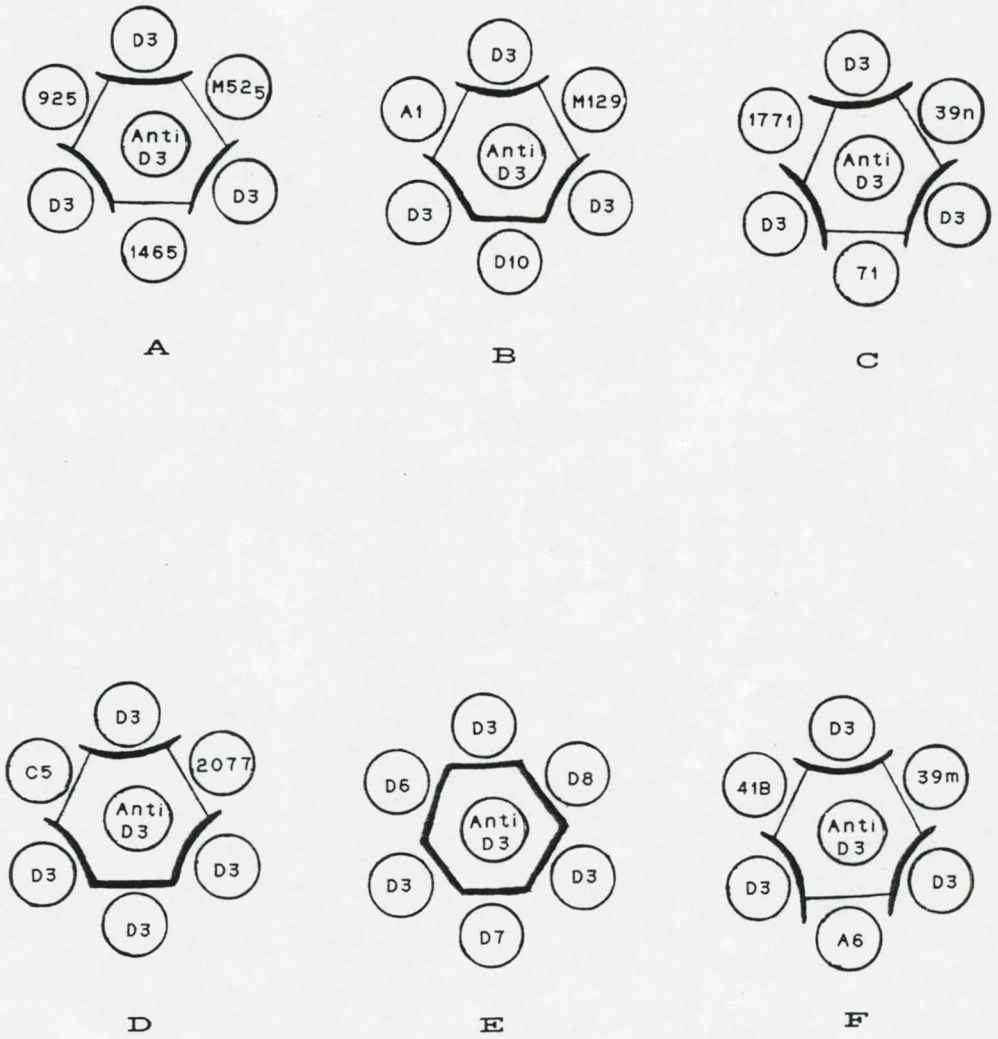


Fig. 15. Ouchterlony immunodiffusion precipitin reactions of type A (minimally washed) cell preparations of *A. tumefaciens* isolates with antiserum against type A cells of isolate D3.

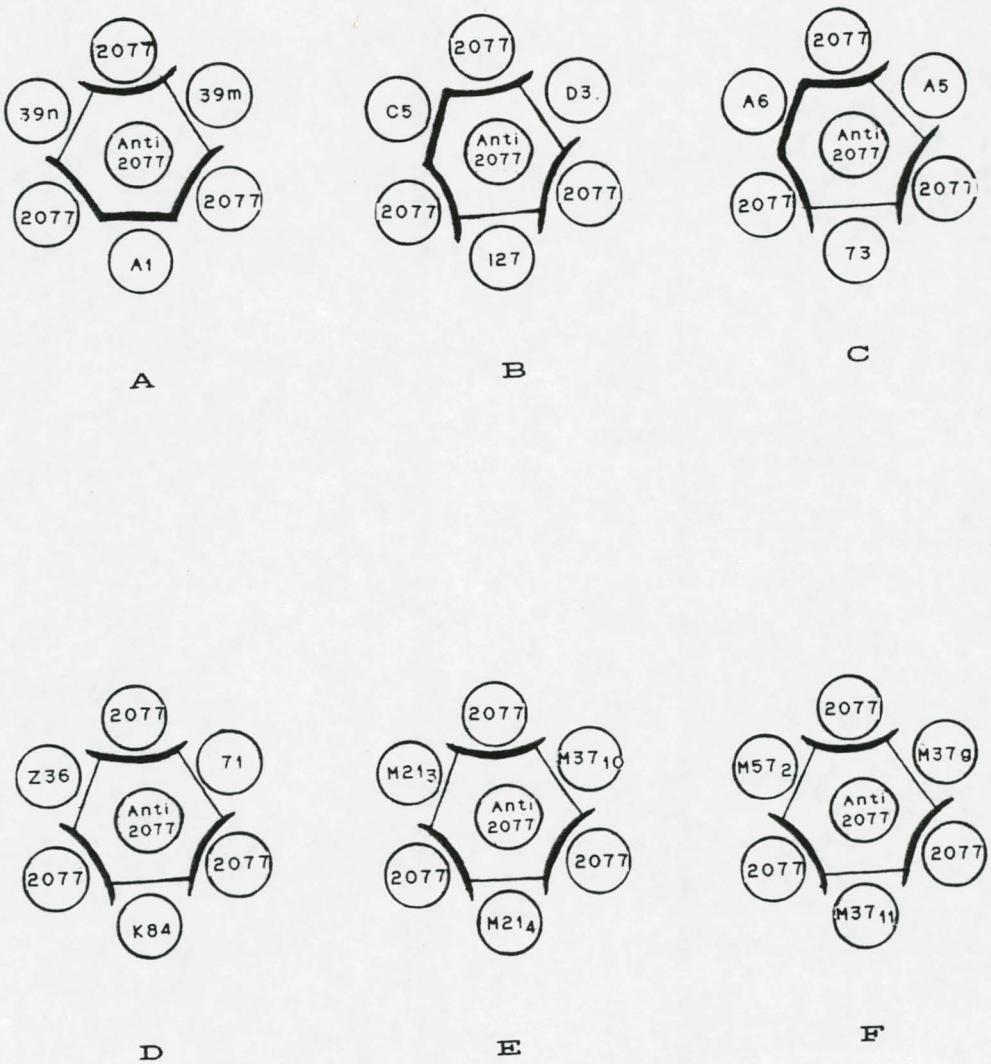


Fig. 16. Ouchterlony immunodiffusion precipitin reactions of type A (minimally washed) cell preparations of *A. tumefaciens* isolates with antiserum against type A cells of isolate 2077.

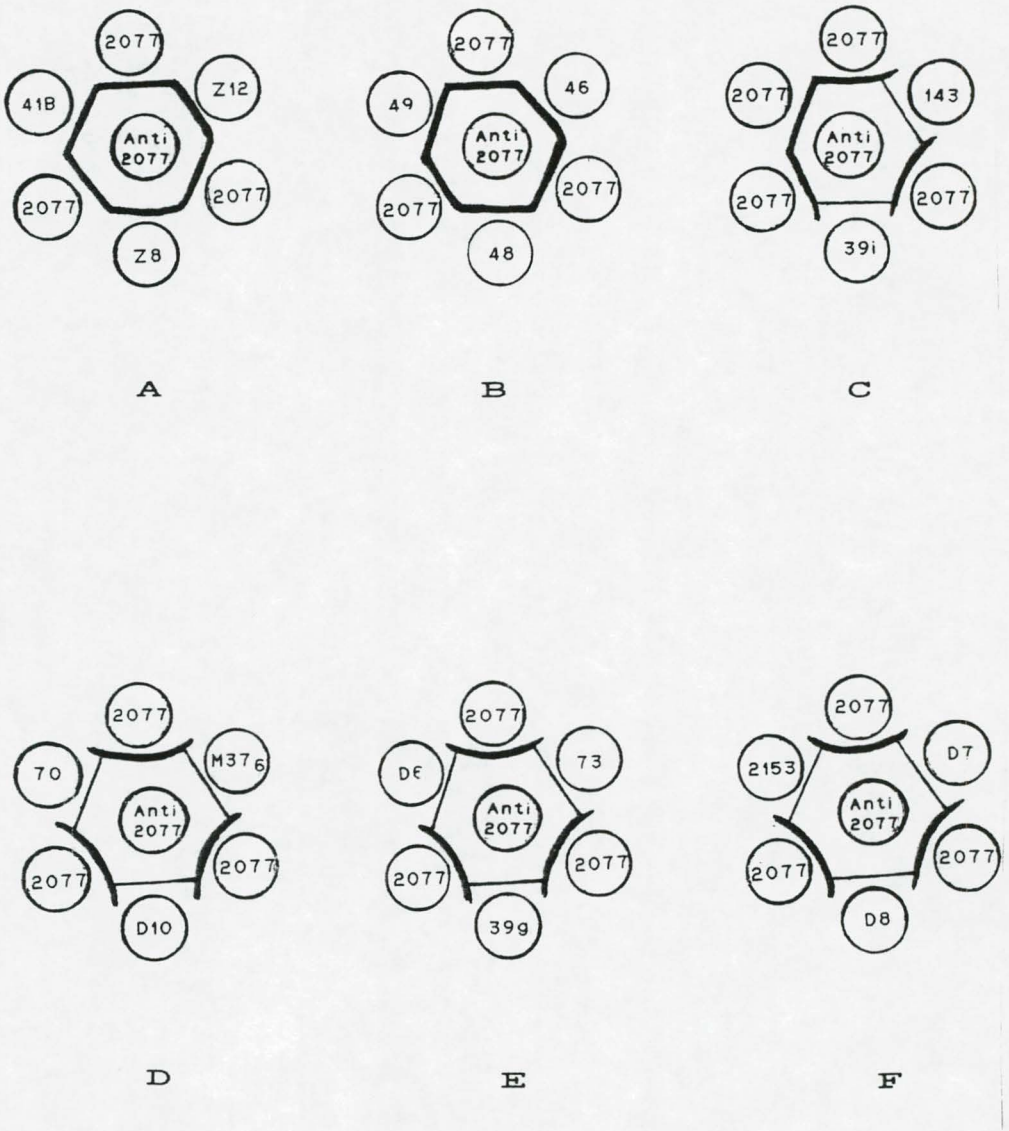


Fig. 17. Ouchterlony immunodiffusion precipitin reactions of type A (minimally washed) cell preparations of *A. tumefaciens* isolates with antiserum against type A cells of isolate 2077.

examination of the antigen wells of the homologous combinations, granular precipitation was observed. Examination of this precipitate under the light microscope (400X magnification) showed a definite clumping (only in the case of the homologous isolates). Thus, it appeared that the biotype 3 isolates 305 and 2221 had no diffusible antigens which could move into the surrounding medium and form a precipitin band with the homologous antiserum.

Ouchterlony immunodiffusion tests with the antisera against type B cells of isolates D3, TT9, $M34_{11}$, 2080, 305 and 2221 versus 'Boivin' antigens extracted from isolates D3, TT9, $M34_{11}$, 2080, 305 and 2221, showed single, sharp and clearly defined precipitin bands in the homologous tests. These bands were much sharper and more clearly defined than those with the phenol-treated cells. It seemed therefore that isolates 305 and 2221 had antigens which were tightly bound to the cell walls and not able to separate from the cells during ordinary immunodiffusion procedures, including phenol- and heat-treatment which were both tested. However, with the more drastic Boivin extraction measures these antigens were removed and readily diffused into the immunodiffusion medium.

Precipitin reactions in Ouchterlony immunodiffusion tests with type B (well washed) cell preparations are illustrated in Fig. 13B and 18-22. Isolate TT9 reacting with homologous TT9 antiserum against type B cells produced a single precipitin band with which the precipitin band of T-37 showed identity (Fig. 18). Bands showing partial identity with that of TT9 with the TT9 antiserum were observed with isolates D10, M51g, $M52_5$, 78, 925, 1465 and 1895. Isolate $M52_3$ produced two parallel bands of partial identity with the band of TT9.

With the antiserum against type B cells of isolate D3, precipitin bands of identity with the band of D3 were obtained with type B cells of the isolates D6, D7 and D8 (Fig. 19). Precipitin bands of partial identity with that of D3 were obtained with isolates A1, A6, C5, 39m, 41B, 1895 and 2077. The agrocin-producing strain K84 also produced a band of partial identity with that of D3.

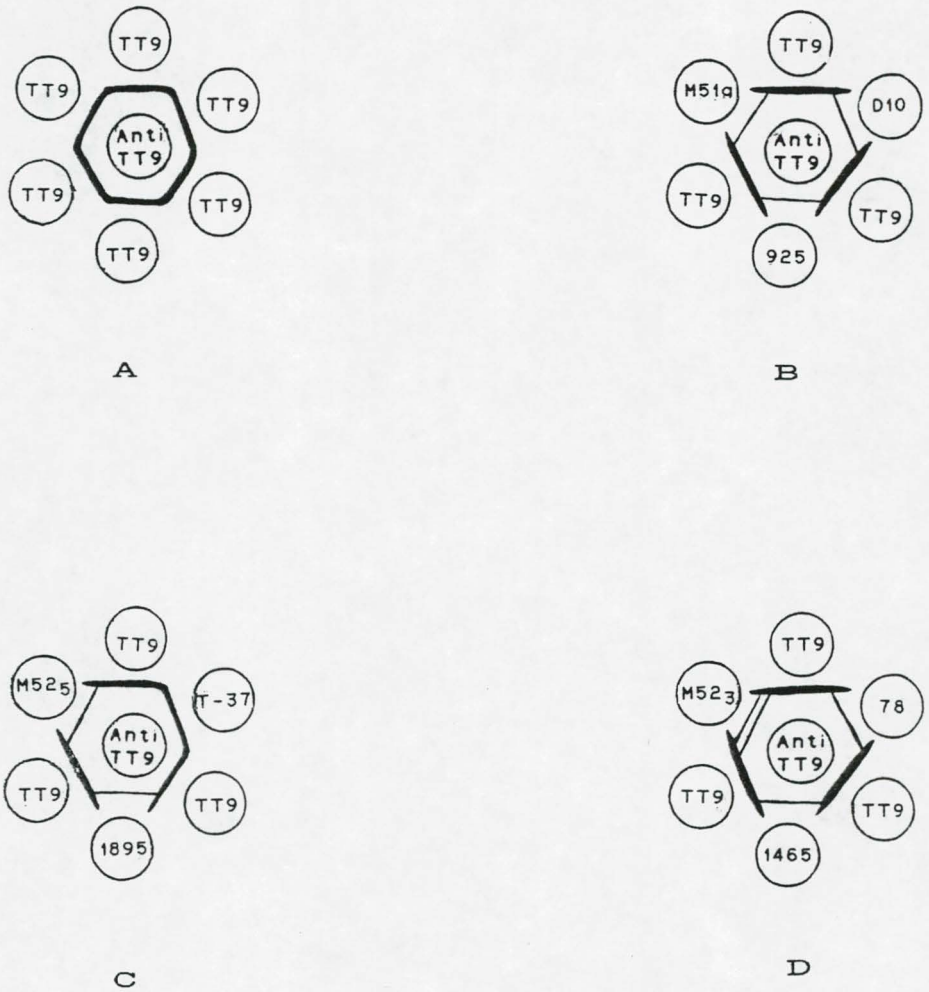


Fig. 18. Ouchterlony immunodiffusion precipitin reactions of type B (well washed) cell preparations of *A. tumefaciens* isolates with antiserum against type B cells of isolate TT9.

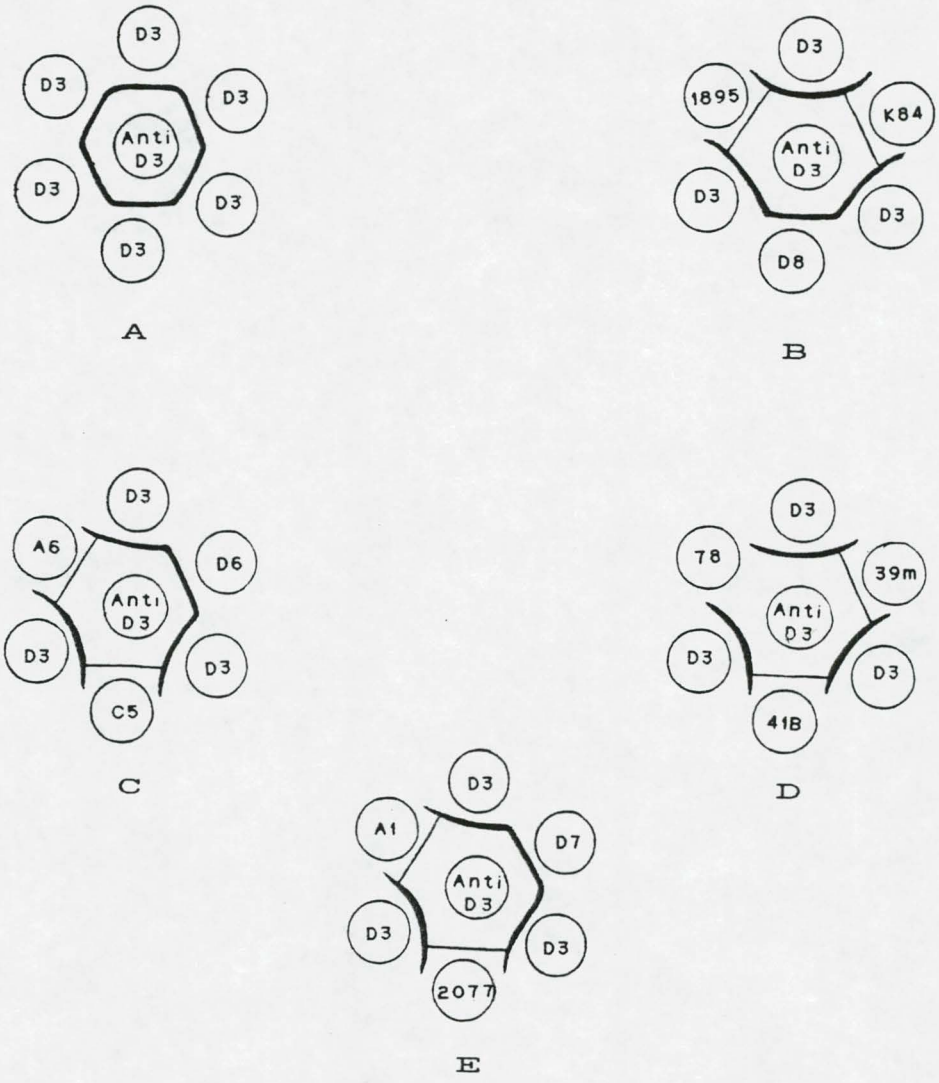


Fig. 19. Ouchterlony immunodiffusion precipitin reactions of type B (well washed) cell preparations of *A. tumefaciens* isolates with antiserum against type B cells of isolate D3.

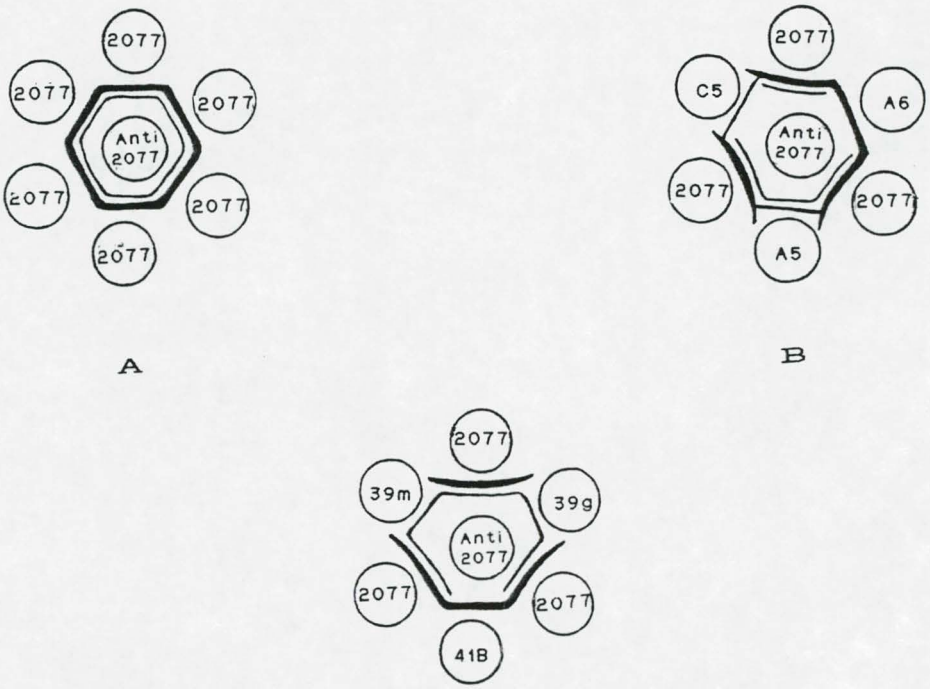


Fig. 20. Ouchterlony immunodiffusion precipitin reactions of type B (well washed) cell preparations of *A. tumefaciens* isolates with antiserum against type B cells of isolate 2077. The reaction of isolate A1 is not shown, but it was similar to that of isolate C5.

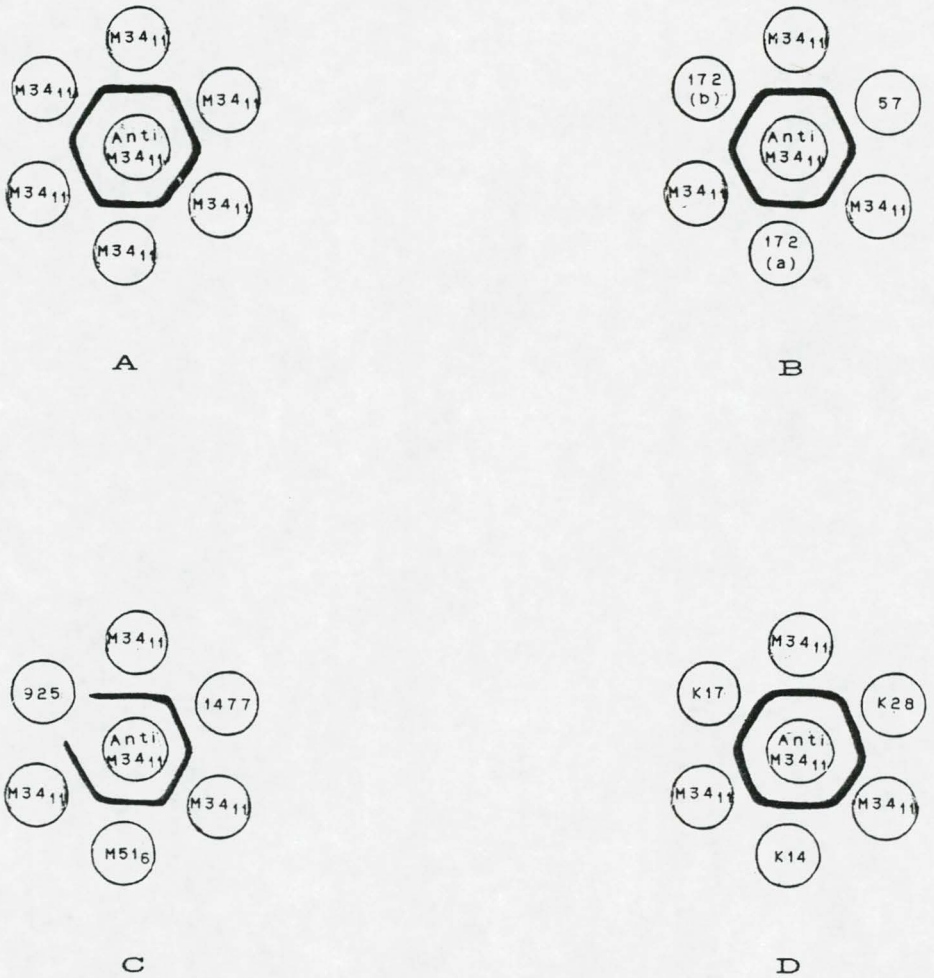


Fig. 21. Ouchterlony immunodiffusion precipitin reactions of type B (well washed) cell preparations of *A. tumefaciens* isolates with antiserum against type B cells of isolate $M34_{11}$.

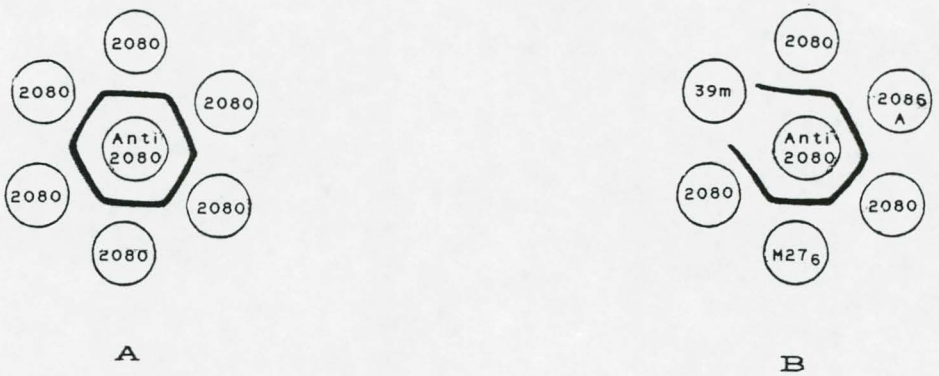


Fig. 22. Ouchterlony immunodiffusion precipitin reactions of type B (well washed) cell preparations of *A. tumefaciens* isolates with antiserum against type B cells of isolate 2080.

Double precipitin bands were obtained in the reaction of antiserum against type B cells of isolate 2077 with homologous type B cells (Fig. 20). The band closest to the antiserum well was designated the B-band and that closest to the antigen well the A-band. The A- band was about twice as thick as the B-band. Isolates A6 and 41B produced a band of identity and isolates A1 and C5 a band of partial identity with the A-band of 2077, whereas isolates 39g and 39m formed a band of identity with the B-band of 2077. With isolate A5, a band of partial identity with the A-band and a band of identity with the B-band of isolate 2077 were observed.

With the antiserum against type B cells of isolate $M34_{11}$, precipitin bands of identity with that of $M34_{11}$ were observed with type B cells of isolates 57, 172(a), 172(b), 1477, K14, K17, K28 and $M51_6$ (Fig. 13B, 21).

Type B cells of only two heterologous isolates (2086A and $M27_6$) reacted with the antiserum against type B cells of isolate 2080, forming precipitin bands of identity with that of 2080 (Fig. 22).

The results obtained in the immunodiffusion tests with type A (minimally washed) cells were very similar to those obtained in the agglutination tests with type A cells. However, immunodiffusion did not produce precipitin bands corresponding to the very low agglutination titres. Serological groupings of the isolates based on the reactions of type A and B cells in the immunodiffusion tests have been made in Tables 12 and 13, respectively. Not as much similarity between immunodiffusion precipitin reactions and agglutination was found in the reactions of the type B (well washed) cell preparations. It appeared that some of the antigens of the well washed cells were tightly bound to the cells and did not readily diffuse into the surrounding medium in the immunodiffusion tests. It seemed that in these cases the reactive antibodies diffused through the medium and precipitated the antigens in the antigen wells. No such problem was noticed with the agglutination tests as the antisera and antigens were in direct contact with one another in a liquid medium.

TABLE 12. Serological grouping of *Agrobacterium* isolates and strains according to immunoprecipitin reactions of type A cell preparations with antisera against type A cells, i.e. based on O/S antigen reactions

Isolate/strain	Band(s) on Ouchterlony plates with antiserum TT9	D3	2077
TT9,T-37	+(I)[a]	-[b]	-
198,2080,2086A,4452, B6,M51g,M52$_3$,	+(PI)[c]	-	-
925,1465,M52$_5$,M129	+(PI)	+(PI)	-
2153,M21$_3$,M21$_4$,M37g, M37$_6$,M37$_{10}$,M37$_{11}$	+(PI)	-	+(PI)
D10	+(PI)	+(I)	+(PI)
1771	-	+(PI)	-
D3,D6,D7,D8	-	+(I)	+(PI)
41B,2077,A1,A6,C5	-	+(PI)	+(I)
39m,39n,71,K84	-	+(PI)	+(PI)
46,47,48,49,Z8,Z12	-	-	+(I)
39i,39g,70,73,143, A5,I27,Z36	-	-	+(PI)
45d,57,78,172(a), 172(b),305,1477, 1671,1887,1895, 2158,2160,2164, 2221,M34$_{11}$,M51$_6$, M52g,M57g,K14,K17, K21,K28,K30,W7,W8, Z13	No reactions detected		

[a]Precipitin band of identity with that of homologous antigen.
[b]No reaction.
[c]Precipitin band of partial identity with that of homologous antigen.

TABLE 13. Serological grouping of *Agrobacterium* isolates and strains according to immunoprecipitin reactions of phenol-treated type B cell preparations with antisera against type B cells, i.e. based on 'deep' (O/D) antigen reactions

Isolate/strain	Bands on Ouchterlony plates with antisera				
	TT9	D3	2077	$M34_{11}$	2080
TT9,T-37	+(I)[a]	-[b]	-	-	-
78,925,1465,D10, $M51g,M52_3,M52_5$	+(PI)[c]	-	-	-	-
1895	+(PI)	+(PI)	-	-	-
D3,D6,D7,D8	-	+(I)	-	-	-
39n,K84	-	+(PI)	-	-	-
A6,41B	-	+(PI)	+(IA)[d]	-	-
A1,C5	-	+(PI)	+(PIA)[e]	-	-
39m	-	+(PI)	+(PIB)[f]	-	-
39g	-	-	+(IB)[g]	-	-
2077	-	-	+(IA)(IB)	-	-
A5	-	-	+(IB)(PIA)	-	-
57,1477,172(a), 172(b),$M34_{11}$, $K14,K28,M51_6$	-	-	-	+(I)	-
2080,2086A	-	-	-	-	+(I)
39i,46,47,48, 49,70,71,73, 143,198,305, 1771,2153,2160, 2164,2221,4452, B6,D286,I27,K21, $K30,M21_3,M21_4$, $M37g,M37_6,M37_{10}$, $M37_{11}$,M129,W7,W8, Z8,Z12,Z36	No reactions detected				

[a]Precipitin band of identity with that of homologous antigen.
[b]No reaction.
[c]Precipitin band of partial identity with that of homologous antigen.
[d]Precipitin band of identity with the A band of homologous antigen.
[e]Precipitin band of partial identity with the A band of homologous antigen.
[f]Precipitin band of partial identity with the B band of homologous antigen.
[g]Precipitin band of identity with the B band of homologous antigen.

The Ouchterlony immunodiffusion precipitin reactions explain some of the titre differences observed in the agglutination tests by indicating whether the agglutination reactions were based on identity or only partial identity of homologous and heterologous antigens reacting with a specific antiserum. However, not all titre differences can be thus explained. For example, as shown in Table 10, minimally washed cells of isolates TT9 and T-37 shared antigens which produced titre differences in the agglutination tests with antiserum TT9. In the immunodiffusion tests, however, these antigens appeared to be similar producing precipitin bands of identity. All the other reactions of heterologous isolates of the agglutination groups O/S-I, O/S-II and O/S-III with the TT9 antiserum were caused by antigens of only partial identity with the antigen(s) of TT9. Isolates D3, D6, D7 and D8 appeared similar from their precipitin reactions with the D3 and 2077 antisera, but showed titre differences in their agglutination reactions with these antisera. However, they showed only partial identity with the antigen(s) of isolate 2077 compared with identity to the antigen(s) of D3.

Antigenic structure of isolates according to agglutination and immunodiffusion tests

Table 14 shows the antigenic structure of most of the *A. tumefaciens* isolates and strains in terms of their antigenic affinities shown by their reactions with the test antisera. With many isolates the superficial O/S and deeper O/D antigens were similar, but many isolates showed loss of O/S antigens and uncovering of O/D antigens following their conversion from type A to type B cell preparations by thorough washing (shaking with glass beads). The highest titre (not necessarily high titre) O-reactions of isolates were regarded as demonstrating main O-antigenic affinities and relatively low titre O-reactions as indicating minor antigenic affinities. With two exceptions, H-reactions were with only a single antiserum.

In Table 15, the isolates and strains have been grouped according to their main O-antigenic affinity(-ies) with the antisera used in agglutination and immunodiffusion tests. A total of 22 different groups could be distinguished. The largest groups were B with 9 isolates, H with 8, D with 6, E with 4 and O with 4 isolates; the remaining groups contained three or fewer isolates. Some of the groups, namely, B, H, J and N, would have to be subdivided if H-reactions were

TABLE 14. Antigenic structure of *Agrobacterium* isolates and strains as indicated by antigenic affinities with available test antisera in agglutination and immunodiffusion tests

Isolate/ strain	O-group and subgroup O/S	O/D	H[a]	Antigenic affinities with antisera Main O[b] Surface O/S	Deeper O/D	Minor O[c] Surface O/S	Deeper O/D
TT9	Ia	Ia	TT9(8)d	TT9(8,I)	TT9(8,I)e	-f	-
T-37	I	Ib	TT9(7)	TT9(6,I)	TT9(I)	-	-
2086A	Ib	XVIIb	TT9(8)	TT9(7,PI)g	2080(7,I)	-	2221(5)
2080	Ic	XVIIa	TT9(7)	TT9(6,PI)	2080(8,I)	-	2221(3)
198	Ic	XVIe	TT9(8)	TT9(6,PI)	-	-	$M34_{11}$(2)
4452	Ic	-	-	TT9(6,PI)	-	-	-
B6	Ic	-	-	TT9(6,PI)	-	-	-
D286	Id	Ic	-	TT9(5)	TT9(6)	-	-
W7	Ie	XIIIb	-	TT9(4)	305(7) 2221(7)	-	D3(2) $M34_{11}$(3)
W8	Ie	-	-	TT9(4)	-	-	-
$M52_3$	Ie	XVIIIb	-	TT9(4,PI)	305(5,PI)	-	-
M51g	If	II	-	TT9(3,PI)	TT9(5,PI)	-	2080(4) 305(4)
$M51_6$	If	XVIb	-	TT9(3,PI)	$M34_{11}$(7)	-	-
1465	IIa	Ib	TT9(7) D3(8)	TT9(6,PI)	TT9(7,PI)	D3(4,PI)	-
925	IIb	IVa	-	TT9(5,PI)	TT9(7,PI)	D3(3,PI)	D3(5)
$M52_5$	IIc	VI	TT9(6)	TT9(5,PI)	TT9(6,PI)	D3(2,PI)	2080(4),2221(3) D3(3),$M34_{11}$(3)
M129	IId	Id	-	TT9(3,PI)	TT9(3)	D3(2,PI)	-
$M37_6$	IIIa	-	-	TT9(4,PI) 2077(4,PI)	-	-	-
M37g	IIIb	-	-	TT9(4,PI) 2077(3,PI)	-	-	-
$M37_{11}$	IIIc	-	-	TT9(4,PI) 2077(2,PI)	-	-	-
$M21_3$	IIId	-	-	TT9(3,PI) 2077(3,PI)	-	-	-
2153	IIId	-	-	TT9(3,PI) 2077(3,PI)	-	-	-
$M37_{10}$	IIIe	-	-	TT9(3,PI) 2077(2,PI)	-	-	-
$M21_4$	IIIe	-	-	TT9(3,PI) 2077(2,PI)	-	-	-
D10	IVa	III	-	D3(5,I) TT9(5,PI)	TT9(7,PI)	2077(2,PI)	2077(4,PI)
1771	Va	Va	TT9(7)	D3(2,PI)	TT9(7)	-	D3(5),305(6)
2160	Va	XIIIc	-	D3(2)	305(6) 2221(5)	-	D3(2) $M34_{11}$(3)
2221	Vb	XIIIa	-	D3(1)	2221(8) 305(8)	-	D3(2),$M34_{11}$(3)
D7	VIa	IXa	-	D3(8,I) 2077(5,PI)	D3(6,I) TT9(6),2077(7)	-	2221(3)
D3	VIb	VIII	-	D3(8,I)	D3(8,I)	2077(3,PI)	2077(5),TT9(3)

TABLE 14 /continued/

Isolate/ strain	O-group and subgroup O/S	O/D	H[a]	Antigenic affinities with antisera Main O[b] Surface O/S	Deeper O/D	Minor O[c] Surface O/S	Deeper O/D
D6	VIc	Xa	-	D3(7,I)	D3(8,I)	2077(2,PI)	305(6),2077(5)
71	VId	IVb	D3(8)	D3(6,PI)	TT9(6)	2077(4,PI)	D3(4)
D8	VIc	IXb	-	D3(6,I)	D3(6,I) TT9(6),2077(6)	2077(3,PI)	2221(3)
41B	VIf	Xb	2077(8)	2077(8,I)	2077(8,IA)	D3(4,PI)	D3(5,PI),305(5)
2077	VIf	XV	2077(8)	2077(8,I)	2077(8,IA,IB)	D3(4,PI)	305(4)
K84	VIg	Xe	-	2077(4,PI) D3(4,PI)	2077(7) 305(7)	-	D3(3,PI)
A6	VIh	Xf	2077(8) D3(8)	2077(8,I)	2077(8,IA)	D3(3,PI)	305(5),D3(2,PI)
C5	VIi	Xc	2077(7)	2077(8,I)	2077(8,PIA)	D3(2,PI)	305(6),D3(4,PI)
39m	VIi	XIa	2077(8)	2077(8,PI)	2077(8,PIB) 305(7)	D3(2,PI)	D3(3,PI)
39n	VIj	VIIa	2077(4)	2077(8,PI)	2077(8) 305(7)	D3(2,PI)	D3(4,PI),TT9(4)
73	VIj	XIVb	2077(7)	2077(8,PI)	2077(7)	D3(2)	-
A1	VIk	VIIb	2077(6)	2077(5,I)	2077(8,PIA)	D3(2,PI)	305(5),D3(3,PI) TT9(2)
39i	VII	-	-	2077(3,PI)	-	D3(2)	-
305	VIm	XVIIIa	-	2077(2) D3(2)	305(8)	-	-
Z12	VIn	Id	2077(8)	2077(8,I)	TT9(3)	D3(1)	-
Z36	VIn	XI	2077(8)	2077(8,PI)	2077(4)	D3(1)	D3(4)
I27	VIo	XIVb	-	2077(6,PI)	2077(7)	D3(1)	-
A5	VIo	XIe	-	2077(6,PI)	2077(7,IB,PIA) 305(7)	D3(1)	D3(3)
48	VII	XIVa	2077(8)	2077(8,I)	2077(8)	-	-
Z8	VIIb	IVd	2077(4)	2077(5,I)	TT9(4)	-	D3(1)
46	VIIb	XIVa	2077(7)	2077(5,I)	2077(8)	-	-
49	VIIb	XIVa	2077(8)	2077(5,I)	2077(8)	-	-
47	VIIb	XIVb	2077(8)	2077(5,PI)	2077(7)	-	-
70	VIIc	Vb	-	2077(4,PI)	D3(5)	-	TT9(4),305(3)
39g	VIIc	XIVc	-	2077(4,PI)	2077(5,IB)	-	-
143	VIId	-	-	2077(3,PI)	-	-	-
172(a)	-	XIIa	-	-	D3(4) $M34_{11}$(5)	-	-
172(b)	-	XIIa	-	-	D3(4) $M34_{11}$(5)	-	-
1895	-	IVc	-	-	TT9(5,PI) D3(4,PI)	-	-
78	-	IVe	-	-	TT9(3,PI) D3(3)	-	-
2164	-	XIIIa	-	-	2221(8) 305(8)	-	D3(2),$M34_{11}$(3)

TABLE 14 /continued/

Isolate/ strain	O-group and subgroup O/S	O/D	H[a]	Antigenic affinities with antisera Main O[b] Surface O/S	Deeper O/D	Minor O[c] Surface O/S	Deeper O/D
$M34_{11}$	-	XVIa	-	-	$M34_{11}(8)$	-	-
K14	-	XVIa	-	-	$M34_{11}(8)$	-	-
K21	-	XVIa	-	-	$M34_{11}(8)$	-	-
K17	-	XVIb	-	-	$M34_{11}(7)$	-	-
K28	-	XVIb	-	-	$M34_{11}(7)$	-	-
K30	-	XVIb	-	-	$M34_{11}(7)$	-	-
$M51_6$	-	XVIb	-	-	$M34_{11}(7)$	-	-
57	-	XVIc	-	-	$M34_{11}(6)$	-	-
1477	-	XVId	-	-	$M34_{11}(4)$	-	-

[a] H-affinity(-ies).
[b] Main O-affinity(-ies).
[c] Minor O-affinity(-ies).
[d] Titre, for example a homologous antigen-antiserum reaction would be 8, half of the homologous titre 7, quarter of the homologous titre 6 etc.
[e] I, precipitin bands of identity.
[f] No reaction.
[g] PI, precipitin bands of partial identity.

TABLE 15. Grouping of *Agrobacterium* isolates and strains according to main O-antigenic affinity(-ies) with available test antisera in agglutination and immunodiffusion tests

Group	Isolates/strains	Main O-antigenic affinity(-ies)
A	TT9,T-37	TT9(I)
B	198,925,1465,4452,B6, M51g,$M52_5$,M129	TT9(PI)
C	D286,W8	TT9
D	2153,$M21_3$,$M21_4$,$M37_6$,M37g, $M37_{10}$,$M37_{11}$	TT9(PI),2077(PI)
E	46,48,49,2077	2077(I)
F	A6,41B	2077(IA)
G	C5,A1	2077(PIA)
H	39i,39g,47,70,73,143, I27,Z36	2077(PI)
I	Z8,Z12	2077(I),TT9
J	39m,39n,A5	2077(PI),305
K	K84,305	305,2077(PI),D3(PI)
L	D3,D6	D3(I)
M	D10	D3(I),TT9(PI)
N	71,78,1771,1895	D3(PI),TT9(PI)
O	D7,D8	D3(I),2077(PI),TT9
P	172(a),172(b)	D3,$M34_{11}$
Q	2160,2221	305,2221,D3
R	W7	305,2221,TT9
S	2164	305,2221
T	$M52_3$	305(PI),TT9(PI)
U	2080,2086A	2080(I),TT9(PI)
V	$M51_6$	$M34_{11}$(I),TT9(PI)
W	57,1477,K14,K17,K21,K28,K30,$M34_{11}$	$M34_{11}$

also taken into account. This has not been done, as the H-affinities of the many isolates which showed no H-agglutination are unknown.

Enzyme-linked immunosorbent assays (ELISA)

In the tests to establish a suitable ELISA procedure, the absorbance values varied greatly with the concentrations of coating gamma globulin, enzyme-linked gamma globulin and bacterial cells (Fig. 3A, 23, 24 and 25). With gamma globulin preparations containing antibodies against type B cells of isolates TT9, D3 or 2077 and the homologous type B bacterial cell preparations, the highest A_{405} values were obtained with 10 ug/ml of the coating globulin preparation, the 1/200 dilution of the enzyme-linked gamma globulin preparation and a bacterial cell concentration of c. 1 X 10^9 cells/ml (Fig. 23, 24 and 25). Only type B (well washed) cell preparations were used in the ELISA study, as these preparations are the most generally used in the ELISA tests and as these ELISA tests were very expensive. The best A_{405} values after the addition of the p-nitrophenylphosphate substrate were obtained with incubation for 12-16 h at 4°C. Very little reaction was found with incubation for 2-4 h at room temperature (22°C). The enzyme-substrate reactions were not stopped with 3 M NaOH as was described by Clark and Adams (1977).

Variations in replicate absorbance values occurred in the outer line of wells due to side-effect phenomona. These artifact discolourations were much more pronounced with the Bios Titertek plates than with the Linbro microtitre plates. Due to these side effects the outer line of wells was not used during the experiments.

Isolates TT9, D3 and 2077 were cross-tested against the anti-TT9, anti-D3 and anti-2077 gamma globulin preparations under the conditions that gave the highest A_{405} values with the homologous bacterial cell preparations. The results of these cross tests (Table 16) showed no cross-reacting O-antigenic determinants among the three isolates, although weak reactions had been shown by isolate D3 with anti-TT9 and anti-2077 antisera in agglutination tests (Table 8).

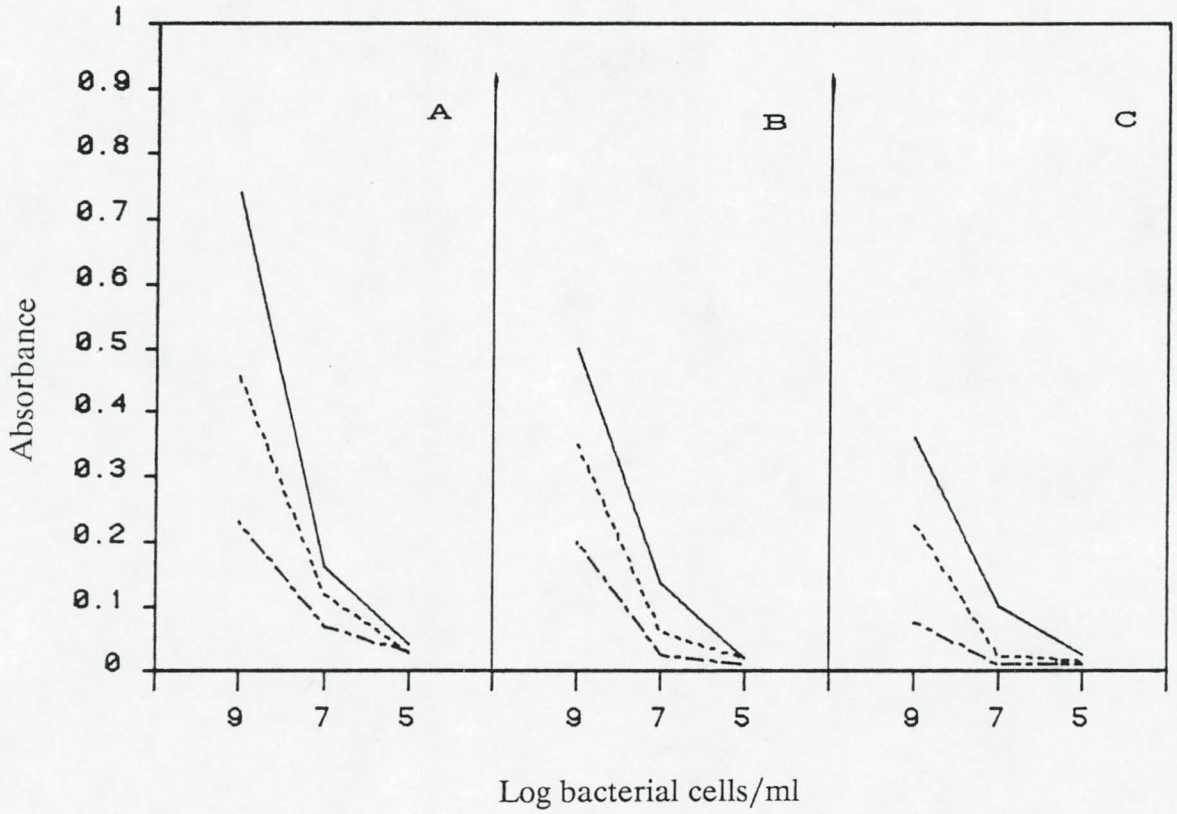


Fig. 23. ELISA absorbance values for type B (well washed) cell preparations of *A. tumefaciens* isolate TT9 diluted in PBS-Tween 20 against enzyme-linked anti-TT9 gamma globulin. Concentration of coating globulin: 10 µg/ml (A); 1 µg/ml (B); 0.1 µg/ml (C). Dilution of enzyme-linked gamma globulin preparation: 1/200 (——); 1/400 (- - - -); 1/800 (— · —). The absorbance of the control without cells was 0.004.

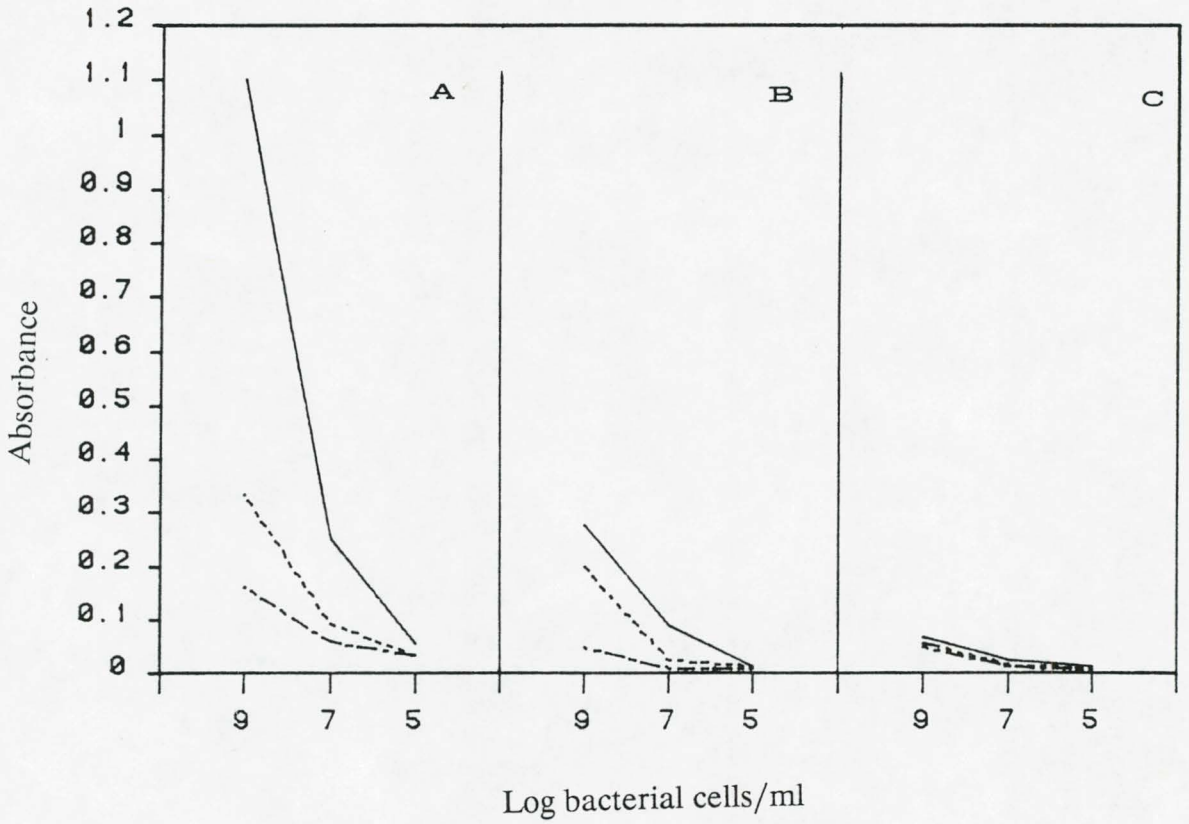


Fig. 24. ELISA absorbance values for type B (well washed) cell preparations of *A. tumefaciens* isolate TT9 diluted in PBS-Tween 20 against enzyme-linked anti-D3 gamma globulin. Concentration of coating globulin: 10 µg/ml (A); 1 µg/ml (B); 0.1 µg/ml (C). Dilution of enzyme-linked gamma globulin preparation: 1/200 (——); 1/400 (- - - -); 1/800 (— - —). The absorbance of the control without cells was 0.004.

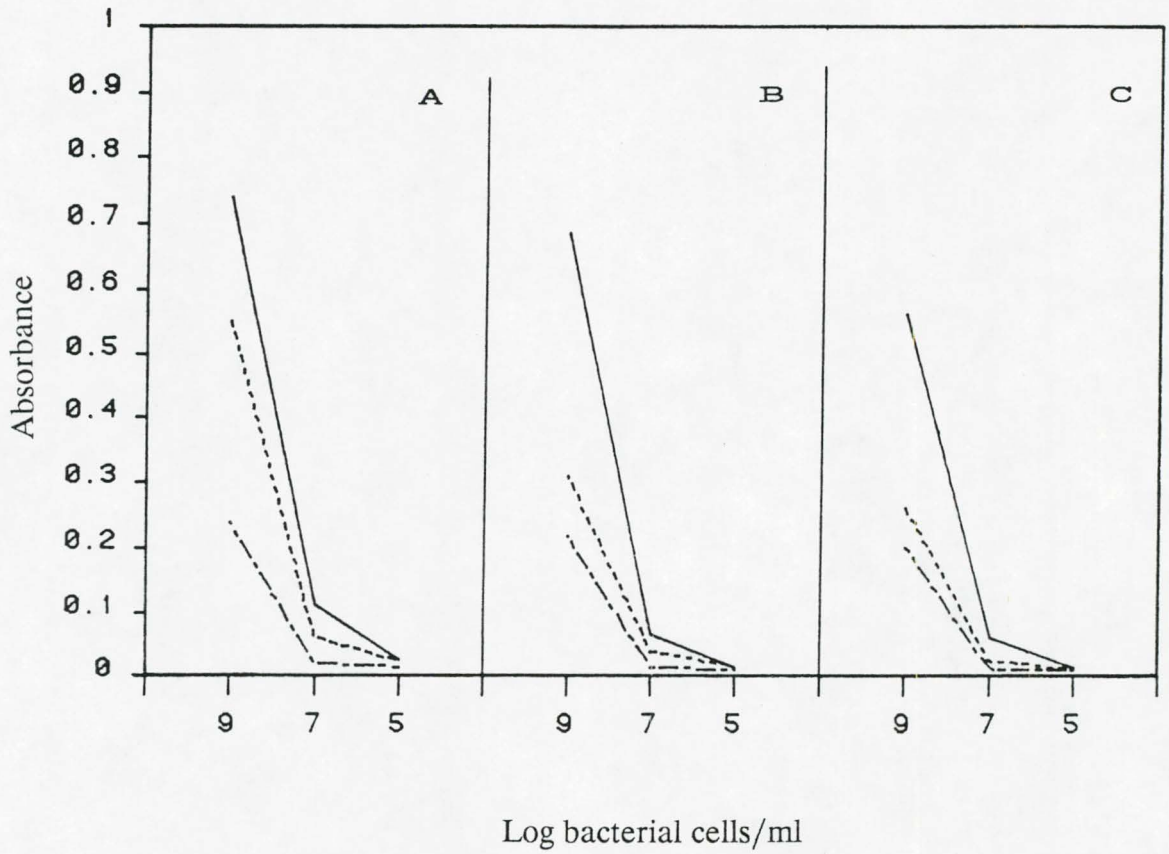


Fig. 25. ELISA absorbance values for type B (well washed) cell preparations of *A. tumefaciens* isolate TT9 diluted in PBS-Tween 20 against enzyme-linked anti-2077 gamma globulin. Concentration of coating globulin: 10 µg/ml (A); 1 µg/ml (B); 0.1 µg/ml (C). Dilution of enzyme-linked gamma globulin preparation: 1/200 (——); 1/400 (- - - -); 1/800 (— — —). The absorbance of the control without cells was 0.004.

TABLE 16. ELISA cross tests with type B (well washed) preparations of a biotype 1 (TT9) and two biotype 2 (D3,2077) *Agrobacterium* isolates and their antibodies (gamma globulin preparations)

Antigen	Absorbance (A_{405})[a] with antibodies against		
	Biotype 1 TT9	Biotype 2 D3	Biotype 2 2077
TT9	0.74	-[b]	-
D3	-	1.1	-
2077	-	-	0.74

[a]Mean of four replicate values.
[b]No or negligible antigen-antibody reaction (absorbance < 0.1).

All isolates were tested against the anti-TT9, anti-D3 and anti-2077 gamma globulin preparations using the same ELISA procedure as in the cross tests. The results and the different serogroups are shown in Table 17.

The negative results include numerous A_{405} absorbance values below 0.1, which were considered too low for the reliable indication of antigen-antibody reactions. (Wells where bound phosphatase enzyme definitely reacted with the substrate produced a yellow colour which was in most cases clearly visible and gave A_{405} values of at least 0.1). The average A_{405} control value in the absence of an antigen-antibody reaction was 0.01. The positive ELISA results corresponded in most cases to strongly positive agglutination results. However, the ELISA tests, like the immunodiffusion tests were less sensitive than the agglutination tests. The absence of ELISA reactions corresponding to low titre agglutinations made the ELISA reactions more specific than the agglutination reactions but provided less information on the overall antigen structure of the isolates. A large number of isolates produced no ELISA reaction.

The reaction of isolates 2080, $M34_{11}$, 2221 and 305 with their homologous antibodies was also investigated by means of the ELISA procedure. However, all tests produced A_{405} values below 0.1, i.e. below the level of definite positive reactions. No further studies were therefore conducted with these antibody preparations.

Relation of serogroups to other strain characteristics

The relation of the main serogroups among the *A. tumefaciens* isolates (Table 15) to biotype, host range and geographical area is summarized in Table 18.

Serogroups A, B, D, P, T, U and V consisted of biotype 1 isolates. All except group P had a main O-antigenic affinity (identity or partial identity) with isolate TT9; however, certain serogroups in other biotypes also had this affinity. Serogroups E, F, G, I, J, L, M and O comprised biotype 2 isolates only; these groups had a main O-antigenic affinity with isolate

TABLE 17. ELISA reactions of *Agrobacterium* isolates and strains (type B cell preparations) with antibodies against type B cells of isolates TT9, D3 and 2077

Isolate/strain (antigen)	Absorbance (A_{405}) of ELISA reaction mixtures with antibodies against TT9	D3	2077
TT9	0.74	-[a]	-
D10	0.53	-	-
T-37	0.50	-	-
172(b)	0.21	-	-
172(a)	0.19	-	-
1465	0.15	-	-
925	0.14	-	-
D286	0.11	-	-
2086A,M129	0.10	-	-
D3	-	1.10	-
D6	-	0.90	-
$M51_6$	-	0.43	-
D7	-	0.70	0.12
D8	-	0.71	0.14
Z36	-	0.33	0.19
A1	-	0.29	0.54
39n	-	0.29	0.47
A5	-	0.24	0.35
2077	-	-	0.74
Z12	-	-	0.45
C5,I27	-	-	0.43
41B	-	-	0.35
39g,49,73	-	-	0.30
A6	-	-	0.29
39m	-	-	0.28
47,48	-	-	0.22
Z8	-	-	0.19
$M52_5$	-	-	0.14
K84	-	-	0.12
46	-	-	0.10
39i,57,70,71,78, 143,198,305,1477, 1771,1895,2080, 2153,2160,2164, 2221,4452,B6,K14, K17,K21,K28,K30, $M21_3$,$M21_4$,$M34_{11}$, $M37g$,$M37_6$,$M37_{10}$, $M37_{11}$,M51g,$M52_3$, W7,W8	No reactions detected		

[a]No or negligible antigen-antibody reaction (absorbance < 0.1).

TABLE 18. Distribution of the different *Agrobacterium* serotypes among isolates of different biotype and from different hosts and geographical areas

Serogroup	No. of isolates	Biotype	Host	Geographical area
A	2	1	*Humulus* sp.	USA[a]
			Juglans sp.	USA
B	8	1	*Dahlia* sp.	RSA[b]
			Prunus sp.	CP[c]
			Pyrus sp.	USA
			Rubus sp.	USA
C	2	1	*Eucalyptus* sp.	TVL[d]
		3	*Vitis* sp.	CP
D	7	1	*Chrysanthemum* sp.	CP
			Prunus sp.	CP
E	4	2	*Prunus* spp.	TVL,OFS[e]
F	2	2	*Prunus* sp.	CP,OFS
G	2	2	*Prunus* sp.	CP
H	8	2	*Prunus* spp.	CP,OFS,TVL
I	2	2	*Prunus* sp.	OFS
J	3	2	*Prunus* sp.	CP,TVL
K	2	2	Soil	AUS[f]
		3	*Vitis* sp.	AUS
L	2	2	*Prunus* sp.	CP
M	1	2	*Prunus* sp.	CP
N	4	1	*Prunus* sp.	TVL
		1	*Vitis* sp.	CP
		2	*Prunus*	CP
		3	*Vitis* sp.	Iran
O	2	2	*Prunus* sp.	CP
P	2	1	*Salix* sp.	OFS
Q	2	3	*Vitis* sp.	CP
R	1	3	*Vitis* sp.	CP
S	1	3	*Vitis* sp.	CP
T	1	1	*Chrysanthemum* sp.	Natal
U	2	1	*Salix* sp.	TVL
V	1	1	*Chrysanthemum* sp.	Natal
W	8	1	*Chrysanthemum* sp.	CP,TVL
			Prunus sp.	OFS
			Vitis sp.	CP

[a]United States of America.
[b]Republic of South Africa.
[c]Cape Province.
[d]Transvaal.
[e]Orange Free State.
[f]Australia.

2077 (identity or partial identity) and/or D3 (identity). Some also had a main affinity with other isolates, including TT9. Serogroups Q, R and S contained only biotype 3 isolates; these serogroups had main O-antigenic affinities with isolates 305 and 2221. Other serogroups, for example, serogroup N, consisted of isolates of all three biotypes, serogroup H of both biotype 1 and 2 isolates, serogroup C of biotype 1 and 3 isolates and serogroup K of biotype 2 and 3 isolates. From these results, serotyping with the antisera used in this study could not be used to assign *A. tumefaciens* isolates to specific biotypes, although a strong reaction with antiserum 2077 would indicate a probable biotype 2 strain and with antiserum 2221 a probable biotype 3 strain.

Some relationship between serogroup and host plant was observed (Table 18). The isolates of serogroups E, F, G, I, L and O were all obtained from *Prunus* spp., those of serogroups P and U from *Salix* sp. and those of serogroup Q from *Vitis* sp. Serogroup D consisted of six biotype 1 isolates from *Chrysanthemum* sp. and one from a *Prunus* sp. However, no host yielded only one serogroup.

Serogroups G, I, L, O, P, Q and U all comprised *Agrobacterium* isolates, within each group, that originated from the same geographical area. Furthermore, the isolates of each of these serogroups were from a single host genus and were of a single biotype. However, other serogroups were more widely distributed, in respect both of geographical area and hosts that they infected.

In Vitro Inhibition of *Agrobacterium tumefaciens* Isolates by Bacteriocinogenic Strains *Agrobacterium radiobacter* K84 and *Agrobacterium tumefaciens* D286

The inhibition of the *A. tumefaciens* isolates challenged by the bacteriocinogenic *A. radiobacter* K84 and *A. tumefaciens* D286 on plates of three different media is recorded in Table 19.

Eleven *A. tumefaciens* biotype 1 isolates (20.8%) showed large (1.8-2.5 cm) clear inhibition zones when tested against *A. radiobacter* strain K84 on SM (Fig. 26A). Biotype 1 isolate 925 showed a smaller zone, of width 1.5 cm, which was not as clearly defined as those of the other biotype 1 isolates. No other biotype 1 strain was sensitive to strain *A. radiobacter* K84 on SM. On YMA, the *A. tumefaciens* biotype 1 isolates inhibited by *A radiobacter* K84 were the same as on SM, except isolates 925 and 1465, that showed no inhibition zone on YMA and 1895 that was not inhibited on SM (Table 19). The width of the large inhibition zones on YMA was approximately half the width of the zones on SM. All *A. tumefaciens* biotype 1 isolates inhibited by *A. radiobacter* K84 on YMA had inhibition zones which were sharp, fairly large (1.0-1.5 cm wide) and clearly defined (Fig. 26C2), except the zone of strain 1895 which was smaller (0.7 cm wide) and not clearly defined. On SEA, as on SM and YMA, the 10 *A. tumefaciens* biotype 1 isolates TT9, T-37, 57, 172(a), 172(b), 198, 1477, 2080, 2086A and 2153 were inhibited by *A. radiobacter* K84 producing large (approximately as large as the zones on SM), clearly defined inhibition zones (Fig. 26D2). These inhibition zones had an average width of 2.1 cm. Eight more *A. tumefaciens* biotype 1 isolates were inhibited by *A. radiobacter* K84 on SEA than on SM, and eight more than on YMA. However, these inhibition zones were small and not clearly defined, having an average width of 0.6 cm.

Only six *A. tumefaciens* biotype 1 isolates (12.5%) were sensitive to *A. tumefaciens* D286 on SM. Their inhibition zones were in general very small (width 0.5-1.0 cm) and not very clearly defined. However, the *in vitro* inhibition of *A. tumefaciens* 172(a) by *A. tumefaciens* D286 on SM showed a well defined, although relatively small inhibition zone (Fig 26B).

TABLE 19. Inhibition of *A. tumefaciens* isolates and strains on three different media by the bacteriocinogenic strains *A. radiobacter* K84 and *A. tumefaciens* D286

A. tumefaciens isolate/strain	Width of inhibition zone (cm)[a] SM K84	SM D286	YMA K84	YMA D286	SEA K84	SEA D286
Biotype 1						
1477	1.8[b]	-[c]	1.4[b]	0.6	2.0[b]	0.5
2080	2.5[b]	-	1.4[b]	0.7	2.5[b]	0.8
2086A	2.5[b]	-	1.5[b]	0.5	2.0[b]	0.5
2153	1.8[b]	-	1.0[b]	0.7	2.0[b]	1.0
57	2.0[b]	-	1.2[b]	0.8	1.9[b]	0.5
TT9	2.0[b]	-	1.3[b]	0.5	2.0[b]	-
T-37	2.3[b]	-	1.2[b]	0.8	2.2[b]	0.4
172(a)	2.0[b]	0.8	1.2[b]	0.7	2.2[b]	0.7
172(b)	2.0[b]	0.5	1.2[b]	0.7	2.0[b]	0.8
198	2.0[b]	-	1.3[b]	0.9	2.0[b]	0.5
925	1.5	-	-	0.6	1.2	0.5
1465	1.8[b]	-	-	0.7	-	0.6
1895	-	-	0.7	0.5	0.7	0.6
$M21_3$	-	-	-	-	0.5	0.5
$M21_4$	-	-	-	-	0.5	0.5
M37g	-	-	-	-	0.5	0.7
45d	-	-	-	-	0.6	-
K14	-	-	-	0.7	0.7	0.7
$M57_2$	-	-	-	-	0.6	-
$M51_6$	-	-	-	0.5	0.8	0.7
$M37_{10}$	-	0.5	-	0.6	-	-
K28	-	1.0	-	0.6	-	0.6
K30	-	0.8	-	0 7	-	0.5
$M52_3$	-	0.8	-	0.5	-	0.6
$M34_{11}$	-	-	-	0.5	-	0.6
M51g	-	-	-	0.5	-	-
K17	-	-	-	0.6	-	-
4452	-	-	-	0.4	-	0.5
K21	-	-	-	0.5	-	0.6
143	-	-	-	0.5	-	0.6
$M37_{11}$	-	-	-	0.5	-	-
$M52_5$	-	-	-	-	-	0.8

TABLE 19. /continued/

A. tumefaciens isolate/strain	Width of inhibition zone (cm)[a] SM K84	SM D286	YMA K84	YMA D286	SEA K84	SEA D286
Biotype 2						
A1	2.0	-	0.5	0.5	0.6	0.6
39g	2.5	-	0.5	-	0.6	-
39i	2.0	-	0.7	-	-	-
C5	1.5	-	1.0	-	-	-
39m	2.2	-	0.8	-	1.2	-
73	1.8	-	0.9	-	1.2	-
39n	1.8	-	0.5	-	0.8	-
41B	2.0	-	-	-	-	-
A5	1.5	-	-	-	-	-
A6	1.8	-	-	-	-	-
2077	-	-	1.4	-	-	-
71	-	-	0.9	-	-	-
D3	-	-	0.6	-	-	-
D10	-	-	0.6	-	-	-
70	-	-	1.4	1.0	-	-
D6	-	-	0.7	-	0.5	-
I27	NT[d]	NT	NT	NT	1.8	0.9
46,47,48,49,Z8, Z12,Z13,Z36	-	-	-	-	-	-

[a] Measured from the outer edge of the colony of the inhibiting strain.
[b]Clear and well defined inhibition zones; all other inhibition zones were faint and not clearly defined.
[c]No inhibition zone.
[d]Not tested.

Fig. 26. Inhibition zones of agrocin K84- and D286-sensitive *A. tumefaciens* isolates.

A. Large inhibition zone with *A. radiobacter* K84 inhibiting *A. tumefaciens* TT9 on Stonier's medium.

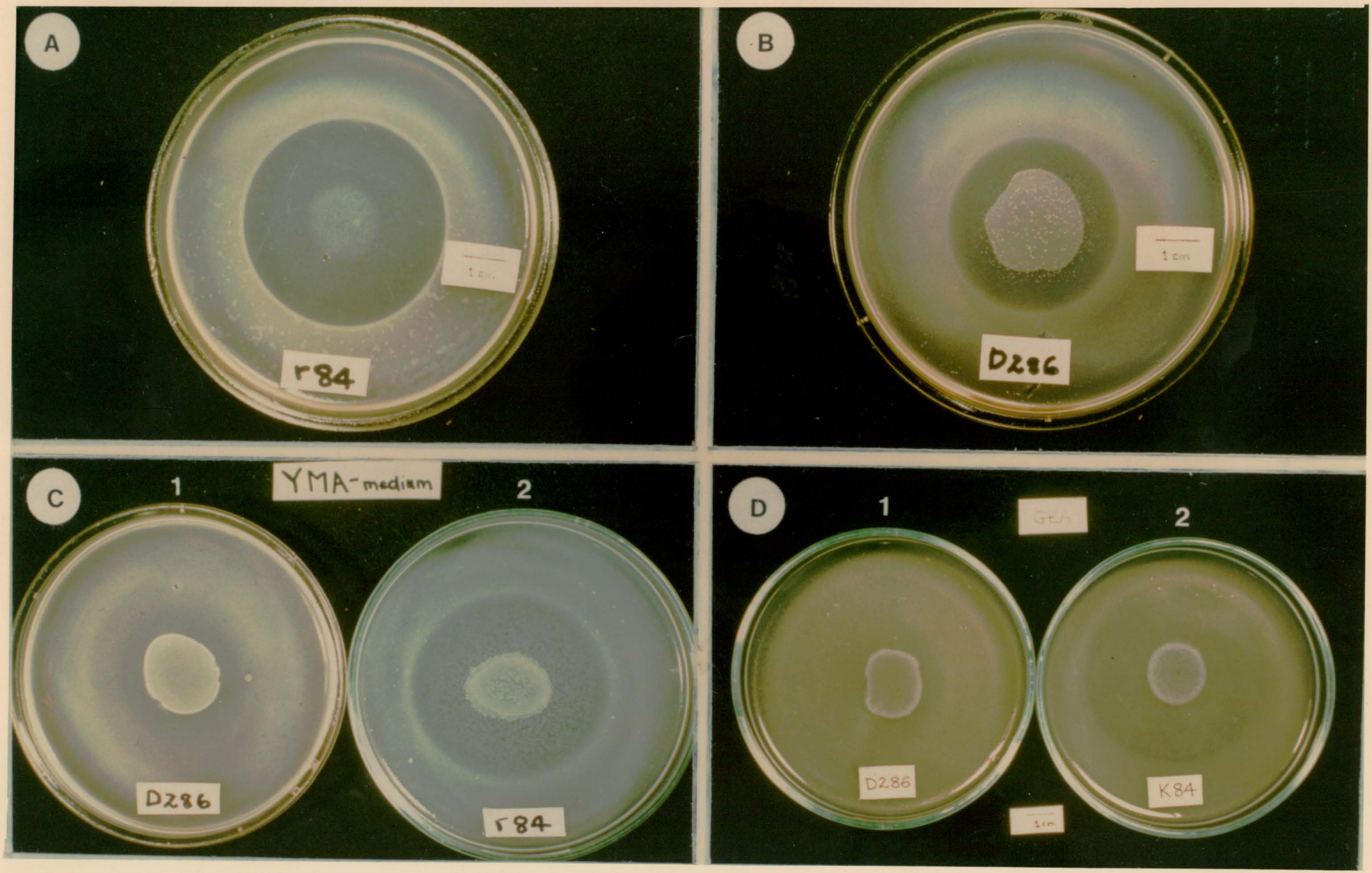
B. Clearly defined small inhibition zone with *A. tumefaciens* D286 inhibiting *A. tumefaciens* 172(a) on Stonier's medium. Note the development of agrocin-resistant colonies in the inhibition zone.

C. **1.** Very faint inhibition zone on YMA medium with *A. tumefaciens* D286 inhibiting *A. tumefaciens* 57.

2. Large inhibition zone on YMA medium with *A. radiobacter* K84 inhibiting *A. tumefaciens* TT9. Large numbers of agrocin-resistant colonies can be seen developing in the inhibition zone.

D. **1.** Faint inhibition zone on SEA medium with *A. tumefaciens* D286 inhibiting *A. tumefaciens* 198.

2. Larger but fainter inhibition zone on SEA medium with *A. radiobacter* K84 inhibiting *A. tumefaciens* TT9.



Most (26) *A. tumefaciens* biotype 1 isolates were sensitive to *A. tumefaciens* D286 on YMA medium. The inhibition zones tended to be small (Fig. 26C1), with average width 0.6 cm. No individual zone was wider than 0.8 cm.

Twenty-five *A. tumefaciens* biotype 1 isolates were inhibited by *A. tumefaciens* D286 on SEA, but the inhibition zones were small (width 0.4-1.0 cm) and not clearly defined (Fig. 26D1). Eight *A. tumefaciens* biotype 1 isolates were inhibited by *A. tumefaciens* D286 on either SEA or YMA but not on both. Differences in the spectra of biotype 1 isolates inhibited by *A. radiobacter* K84 and *A. tumefaciens* D286, respectively, were indicated by the findings that 12 *A. tumefaciens* biotype 1 isolates susceptible to *A. tumefaciens* D286 were not discernably affected by *A. radiobacter* K84.

Ten biotype 2 isolates were inhibited by *A. radiobacter* K84 on SM. These inhibition zones were relatively poorly defined and not always easily detectable. However, they ranged from 1.5 to 2.5 cm with an average width of 1.9 cm. More (13) *A. tumefaciens* biotype 2 isolates were sensitive to *A. radiobacter* K84 on YMA than on SM, with six isolates which were not inhibited by K84 on SM, being inhibited on YMA. The inhibition zones on YMA had an average width of only 0.8 cm (about half the width of those on SM) and were not sharp and clearly defined. Fewer biotype 2 isolates (7) were inhibited by *A. radiobacter* K84 on SEA than on SM or YMA, and these included isolate I27 which was not tested on SM or YMA. The widths of the inhibition zones ranged from 0.6 to 0.8 cm.

No inhibition zones were shown by biotype 2 isolates with *A. tumefaciens* D286 on SM. Only two *A. tumefaciens* biotype 2 isolates (A1,70) were sensitive to *A. tumefaciens* D286 on YMA medium. The inhibition zones produced by these isolates were small to moderate in size (0.5 and 1.0 cm). Also, only two *A. tumefaciens* biotype 2 isolates, A1 and I27, were inhibited by *A. tumefaciens* D286 on SEA (isolate A1 was inhibited on YMA but not on SM, whereas isolate I27 was not tested on YMA and SM). Fourteen *A. tumefaciens* biotype 2 isolates inhibited by *A. radiobacter* K84 on at least one of the media were unaffected by *A. tumefaciens* D286 on any of the test media.

None of the *A. tumefaciens* biotype 3 isolates tested was inhibited by either *A. radiobacter* K84 or *A. tumefaciens* D286 on any of the three media used for the tests.

The inhibition zones produced by isolates of *A. tumefaciens* in response to *A. radiobacter* K84 and *A. tumefaciens* D286 (Table 19) could be classified as large, moderate and small according to the system shown in Table 20. It is clear from Table 20 that the zone sizes on YMA differed noticeably from those on the other three media. In Tables 21 and 22, inhibited isolates have been grouped according to the pattern of inhibition, including zone width, shown in Table 19. Only 10 *A. tumefaciens* biotype 1 isolates showed large or moderate inhibition zones (with *A. radiobacter* K84) on all three test media (Table 21). However, 20 of the biotype 1 isolates were inhibited by *A. radiobacter* K84 on at least one of the test media. Twelve *A. tumefaciens* isolates (all biotype 1) were not inhibited by *A. radiobacter* K84 on any of the three media. Seventeen of the 25 biotype 2 isolates were inhibited by *A. radiobacter* K84 on at least one of the test media.

In the case of inhibition by *A. tumefaciens* D286 (Table 22), 12 different inhibition zone patterns could be distinguished among the biotype 1 isolates and at least three among the biotype 2 isolates. The sizes of the zones produced by individual isolates on the three different media differed considerably (with the exception of *A. tumefaciens* biotype 1 isolate $M52_3$); however, *Agrobacterium tumefaciens* D286 caused no large inhibition zones to be produced by the *A. tumefaciens* biotype 1 and 2 isolates on any of the media tested. Many of the *A. tumefaciens* isolates showing moderate or small inhibition zones with *A. tumefaciens* D286 on YMA and SEA showed no inhibition on SM. Twenty-four isolates, of which 22 were biotype 2, showed no inhibition by *A. tumefaciens* D286 on any of the three media. The sizes of the zones of inhibition produced by the pathogenic *A. tumefaciens* isolates in response to *A. radiobacter* K84 and *A. tumefaciens* D286 on the three different media provide further evidence (apart from different spectra of inhibited isolates) of differences in agrocin production by the two inhibitory isolates. Thus, the agrocin of *A. radiobacter* K84 produced mainly large and

TABLE 20. Classification of zones of inhibition produced by strains of *A. tumefaciens* in response to *A. radiobacter* K84 and *A. tumefaciens* D286 on three different media, according to width

Inhibition zone type		Width of zone (cm) on SM	YMA	SEA
Large	(+++)	1.8-2.5	1.1-1.5	1.8-2.5
Moderate	(++)	1.0-1.7	0.6-1.0	1.0-1.7
Small	(+)	0.2-0.9	0.1-0.5	0.2-0.9

TABLE 21. Grouping of *A. tumefaciens* isolates according to inhibition responses to *A. radiobacter* K84 on SM, YMA and SEA

Isolates	Sero-group(s)[a]	Inhibition zone type on SM	YMA	SEA
Biotype 1				
57,172(a),172(b), 198,1477,2080, 2086A,TT9,T-37	A,B,P,U,W	+++	+++	+++
2153	D	+++	++	+++
925	B	++	-	++
1895	N	-	+	+
$M21_3$,$M21_4$,M37g, $M51_6$,$M57_2$,45d,K14	D,V,W	-	-	+
$M34_{11}$,$M37_{10}$,$M37_{11}$, M51g,$M52_3$,$M52_5$, K17,K21,K28,K30, 143,4452	B,D,T,W	-	-	-
Biotype 2				
39m,73	H,J	+++	++	++
39i	H	+++	++	-
A1,39g,39n	G,H,J	+++	+	+
A6,41B	F	+++	-	-
C5	G	++	++	-
A5	J	++	-	-
D6	L	-	++	+
70,2077	E,H	-	+++	-
71,D3,D10	L,M,N	-	++	-
46,47,48,49, Z8,Z12,Z13,Z36	E,H,I	-	-	-
I27	H	NT	NT	NT

[a]Not known for every isolate in certain inhibition response groups.

TABLE 22. Grouping of *A. tumefaciens* isolates according to inhibition responses to *A. tumefaciens* D286 on SM, YMA and SEA

Isolates	Sero-group(s)[a]	Inhibition zone type on SM	YMA	SEA
Biotype 1				
K28	W	++	++	+
172(a),172(b),K30	P,W	+	++	+
$M52_3$	T	+	+	+
$M37_{10}$	D	+	++	-
2153	D	-	++	++
57,198,925,1477, 2080,T-37,K14	A,B,U,W	-	++	+
143,1465,2086A, $1895,M51_6,M34_{11}$	B,N,U,V,W	-	+	+
K17	W	-	++	-
$TT9,M51g,M37_{11}$	A,B	-	+	-
4452,K21	B,W	-	+	+
$M21_3,M21_4,M37g,M52_5$	B,D	-	-	+
$45d,M57_2$		-	-	-
Biotype 2				
A1	G	-	+	+
70	H-	++	-	
46,47,48,49,71,73, 2077,D3,D6,D10,39g, 39i,39m,39n,41B,A5, A6,C5,Z8,Z12,Z13,Z36	E,F,G,H, I,J,L,M,N	-	-	+
I27	H	NT	NT	+

[a]Not known for every isolate in certain inhibition groups.

moderate inhibition zones, whereas that of *A. tumefaciens* D286 produced only moderate and small inhibition zones.

There were no indications that particular inhibition patterns by either *A. radiobacter* K84 or *A. tumefaciens* D286 were associated with particular serogroups among the *A. tumefaciens* isolates (Tables 21,22).

The numbers of *A. tumefaciens* biotype 1 and 2 isolates inhibited by *A. radiobacter* K84 and *A. tumefaciens* D286 on SM, YMA and SEA are tabulated in Table 23. Marked effects were shown by each of the three variables, namely, biotype, inhibitory strain and medium. Important data which can be deduced from Table 23 are the percentages of *A. tumefaciens* isolates of each of the biotypes which were inhibited on at least one of the three media by the inhibitory strains. Thus, of the 32 *A. tumefaciens* biotype 1 isolates, 63% were inhibited by *A. radiobacter* K84 and 94% by *A. tumefaciens* D286. Of the 25 biotype 2 isolates, 68% were inhibited by *A. radiobacter* K84 and only 12% by *A. tumefaciens* D286. No biotype 3 isolates were inhibited on any of the media by either *A. radiobacter* K84 or *A. tumefaciens* D286

Colonies developing inside the inhibition zones of *A. tumefaciens* isolates inhibited by either *A. radiobacter* K84 and *A. tumefaciens* D286 produced no tumorous outgrowths when inoculated into tobacco and datura stems. All colonies selected from outside these inhibition areas proved to be pathogenic when inoculated into the stems on both tobacco and datura.

In Vivo Inhibition of *Agrobacterium tumefaciens* Isolates by Bacteriocinogenic Strains *Agrobacterium radiobacter* K84 and *Agrobacterium tumefaciens* D286

Application of pathogenic isolates 24-30 h after bacteriocinogenic strains

Results of the experiments with *A. radiobacter* K84 and *A. tumefaciens* D286 applied to the tobacco and datura test plants before the pathogens are shown in Fig. 27 and Appendix Tables 1 and 2.

TABLE 23. Numbers of isolates of three biotypes of *A. tumefaciens* inhibited *in vitro* by agrocinogenic *A. radiobacter* K84 and *A. tumefaciens* D286 on three different media (SM, YMA and SEA)

Biotype	Total number of isolates tested	Number of isolates inhibited on/by SM		YMA		SEA		Number of isolates inhibited on any of the media by	
		K84	D286	K84	D286	K84	D286	K84	D286
1	32	12	6	11	26	19	25	20	30
2	25	10[a]	0[a]	13[a]	2[a]	7	2	17	3
3	8	0	0	0	0	0	0	0	0
1-3 (total)	65	22	6	24	28	26	27	37	33

[a]These values exclude isolate I27, for which no results were obtained with SM and YMA; the other biotype 2 values indicate isolate I27.

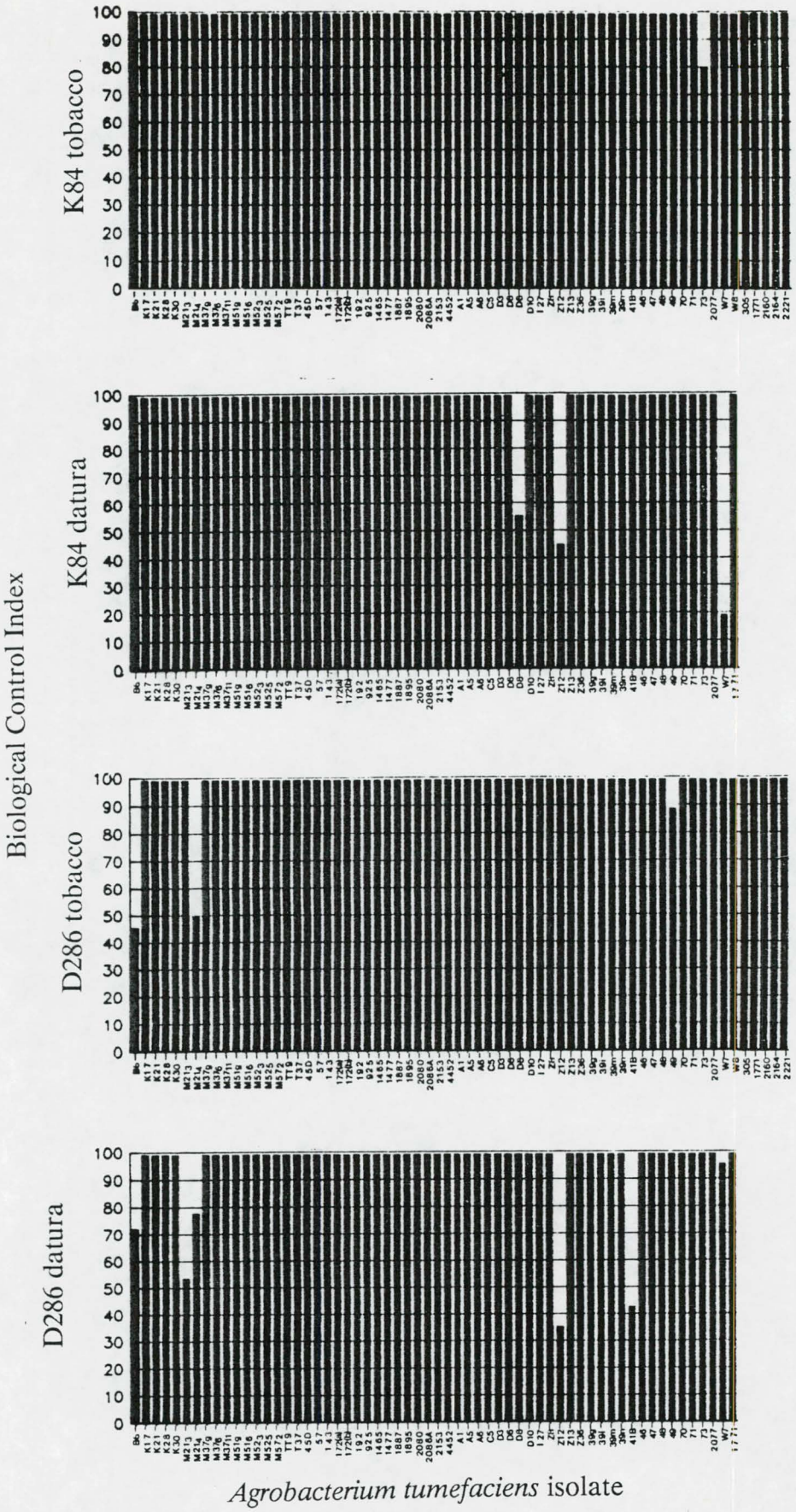


Fig. 27. *In vivo* control of isolates of *A. tumefaciens* by the control strains, *A. radiobacter* K84 and *A. tumefaciens* D286, where the control strain was applied 24-30 h before the isolate.

In the experiment using *A. radiobacter* K84 on tobacco the percentages of control (Table. 24) and the BCI values (Fig. 27; Appendix Tables 1 and 2) were very high and almost all pathogenic isolates (98.5%) were completely controlled, except biotype 2 isolate 73. Where *A. radiobacter* K84 was applied to datura nearly all pathogenic isolates (95%) were controlled, except biotype 2 isolates D8 and Z12 and biotype 3 isolate W7. On datura, *A. tumefaciens* biotype 3 isolates, except isolates W7 and 1771 (Appendix Table 2, pathogen control), failed to produce tumours and were not taken into consideration in any of the *in vivo* inhibition studies. However, on tobacco all *A. tumefaciens* biotype 3 isolates produced tumours on the pathogen control (Appendix Table 1).

With the application of *A. tumefaciens* D286 to tobacco, 92.3% of the pathogenic isolates were controlled, but not biotype 1 isolates B6 and $M21_4$ and biotype 2 isolate 49. On datura, *A. tumefaciens* D286 controlled 90% of the pathogenic isolates. The isolates not completely controlled were biotype 1 isolates B6, $M21_3$ and $M21_4$, biotype 2 isolates Z12 and 41B and biotype 3 isolate W7 (Fig. 27; Table 24; Appendix Tables 1 and 2).

On tobacco all *A. tumefaciens* biotype 3 isolates were completely controlled by *A. radiobacter* K84 (Fig. 27; Appendix Tables 1 and 2).

Application of bacteriocinogenic strains 24-30 h after the pathogenic isolates

Results of the experiments with *A. radiobacter* K84 and *A. tumefaciens* D286 applied to the tobacco and datura test plants after the pathogens are shown in Fig. 28 and Appendix Tables 1 and 2.

These results indicate very low percentages of control (Table 24). Where *A. radiobacter* K84 was applied to tobacco only four (6.2%) of the pathogenic isolates were completely controlled. These isolates were biotype 1 isolates M37g and $M52_3$; biotype 2 isolate 48 and biotype 3 isolate 2164 (Fig. 28; Appendix Table 1). On datura, *A. radiobacter* K84 completely controlled

TABLE 24. Percentages of control with the application of pathogenic isolates 24-30 h after and before the bacteriocinogenic strain

Test plants	Pathogens 24-30 h after control strain		Pathogens 24-30 h before control strain	
	K84	D286	K84	D286
Tobacco	98.5	95.4	6.2	7.7
Datura	95.0	90.0	3.3	1.7

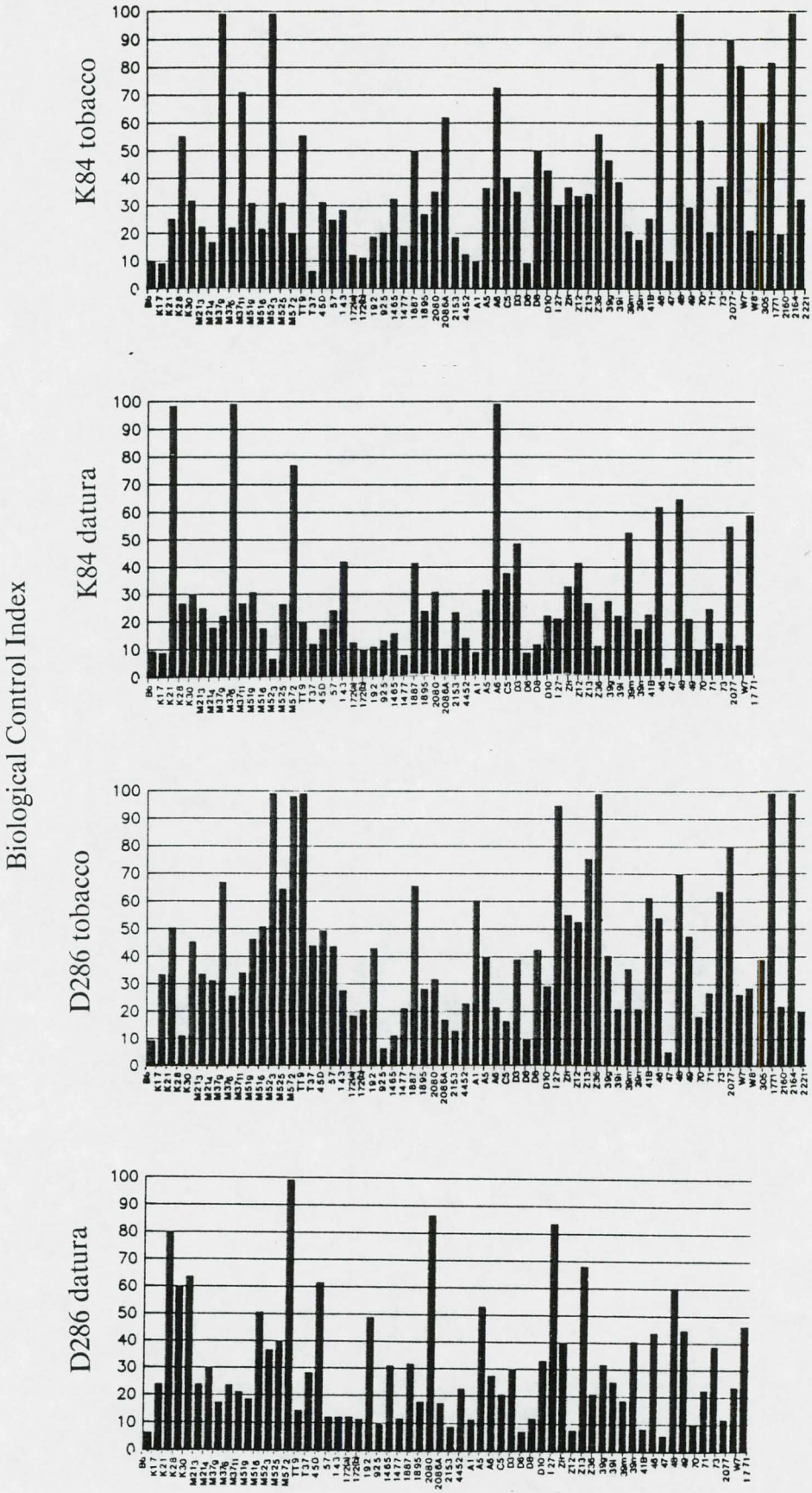


Fig. 28. *In vivo* control of isolates of *A. tumefaciens* by the control strains, *A. radiobacter* K84 and *A. tumefaciens* D286, where the control strain was applied 24-30 h after the isolate.

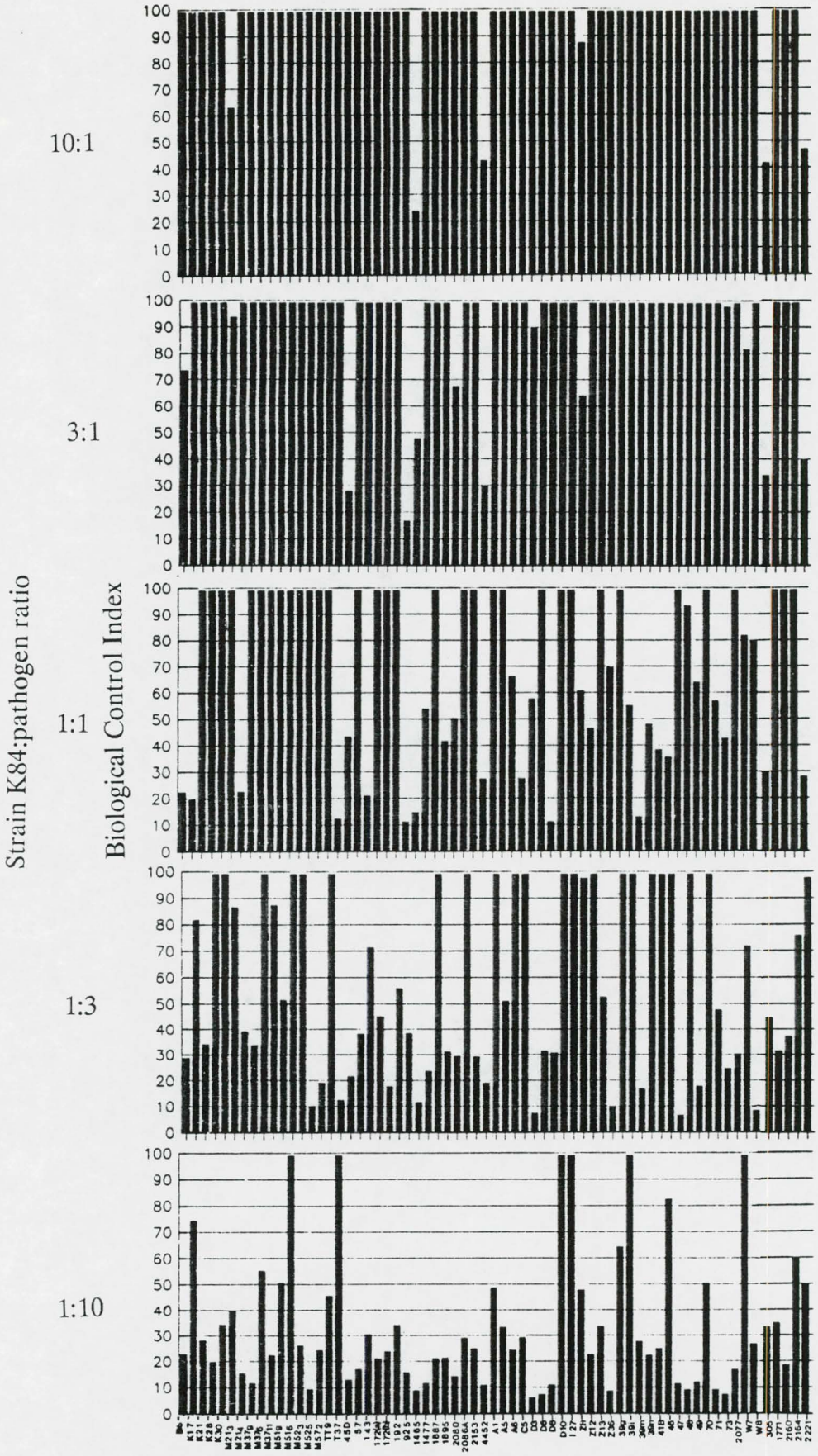
only two (3.3%) of the pathogenic isolates. These pathogens were biotype 1 isolate M37g and biotype 2 isolate A6 (Fig. 28; Appendix Table 2).

Where *A. tumefaciens* D286 was applied to tobacco, only five (7.7%) of the pathogenic isolates were completely controlled (Fig. 28; Table 24: Appendix Table 1). These pathogens were biotype 1 isolates $M52_3$ and TT9; biotype 2 isolate Z36 and biotype 3 isolates 1771 and 2164 (Fig. 28; Appendix Table 1). The application of *A. tumefaciens* D286 to datura controlled only one (1.7%) of the pathogens, namely, biotype 1 isolate $M57_2$.

There have been reports (Garrett, 1979) of amplification of tumour formation following treatment of plants with *A. radiobacter* K84, in other words larger tumours arising after treatment with *A. radiobacter* K84 when compared to tumours on the pathogen controls. This phenomenon was also observed in the present studies where *A. radiobacter* K84 and *A. tumefaciens* D286 were applied after the pathogenic isolates, for example, on tobacco where *A. radiobacter* K84 was applied after the pathogenic biotype 1 isolates K17, T-37, and 172(a) and the biotype 2 isolate Z8 (Appendix Table 1). A similar effect was observed on datura where *A. radiobacter* K84 was applied after the biotype 1 isolates K17, $M21_4$, M51g, $M52_3$, T-37, 172(a), 172(b) and 198; the biotype 2 isolate A1 and the biotype 3 isolate W7 (Appendix Table 2). Applying *A. tumefaciens* D286 on datura after pathogenic biotype 1 isolates $M51_6$ and 172(a) and biotype 2 isolates Z12 and 41B also resulted in the amplification of tumours (Appendix Table 2). Amplification was also observed when *A. tumefaciens* D286 was applied on tobacco after biotype 1 isolates K28, 172(a) and 925 and the biotype 2 isolates Z8 and 39i (Appendix Table 1).

Simultaneous application of pathogenic isolates and bacteriocinogenic strains in different ratios

Results of the experiments with *A. radiobacter* K84 or *A. tumefaciens* D286 applied to tobacco and datura simultaneously with the pathogenic isolates in different ratios are shown in Fig. 29, 30, 31 and 32, as well as Appendix Tables 1 and 2.



Agrobacterium tumefaciens isolate

Fig. 29. *In vivo* control of isolates of *A. tumefaciens* by *A. radiobacter* K84 on tobacco seedlings inoculated with suspensions containing K84 and pathogen cells in different ratios.

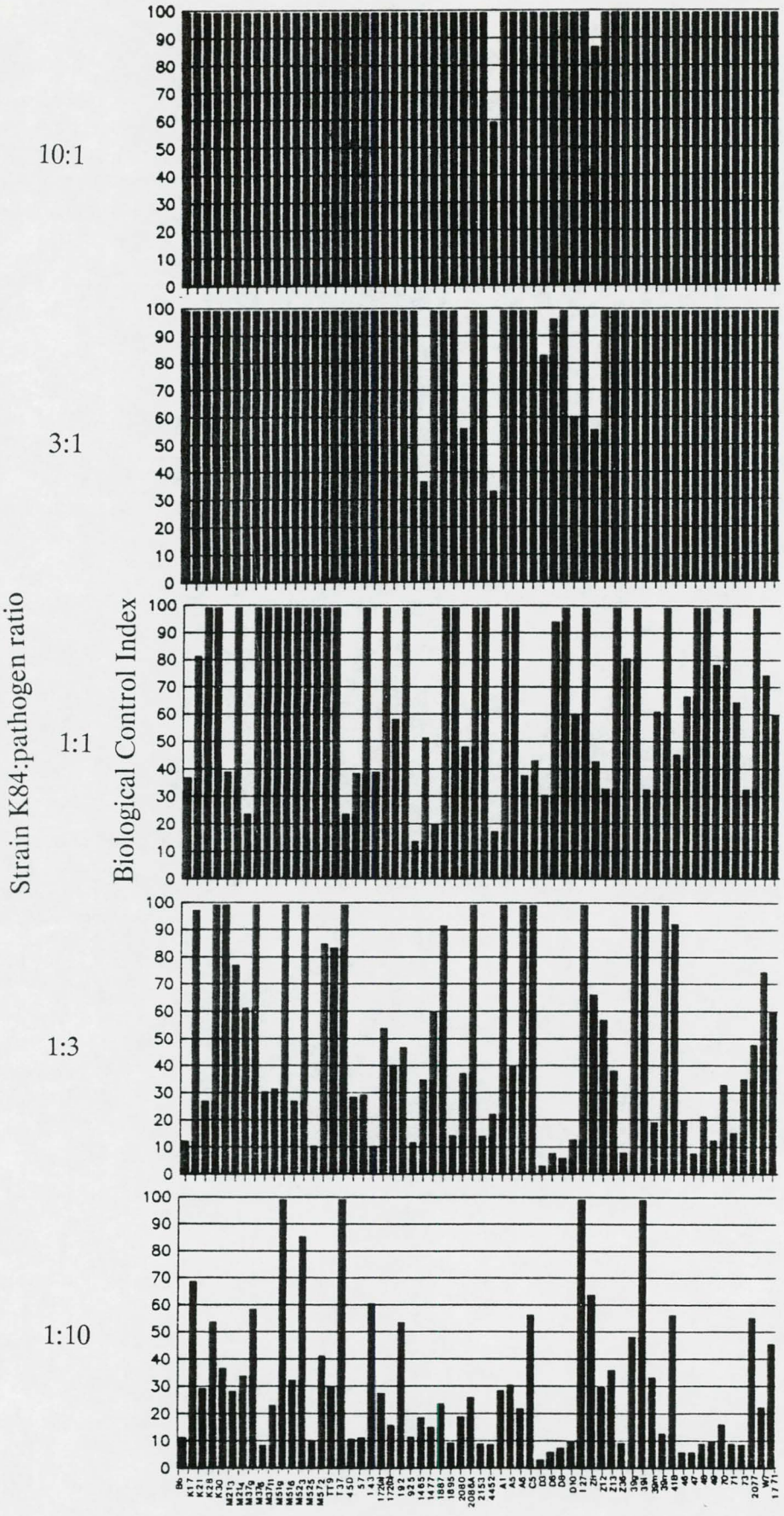


Fig. 30. *In vivo* control of isolates of *A. tumefaciens* by *A. radiobacter* K84 on datura seedlings inoculated with suspensions containing K84 and pathogen cells in different ratios.

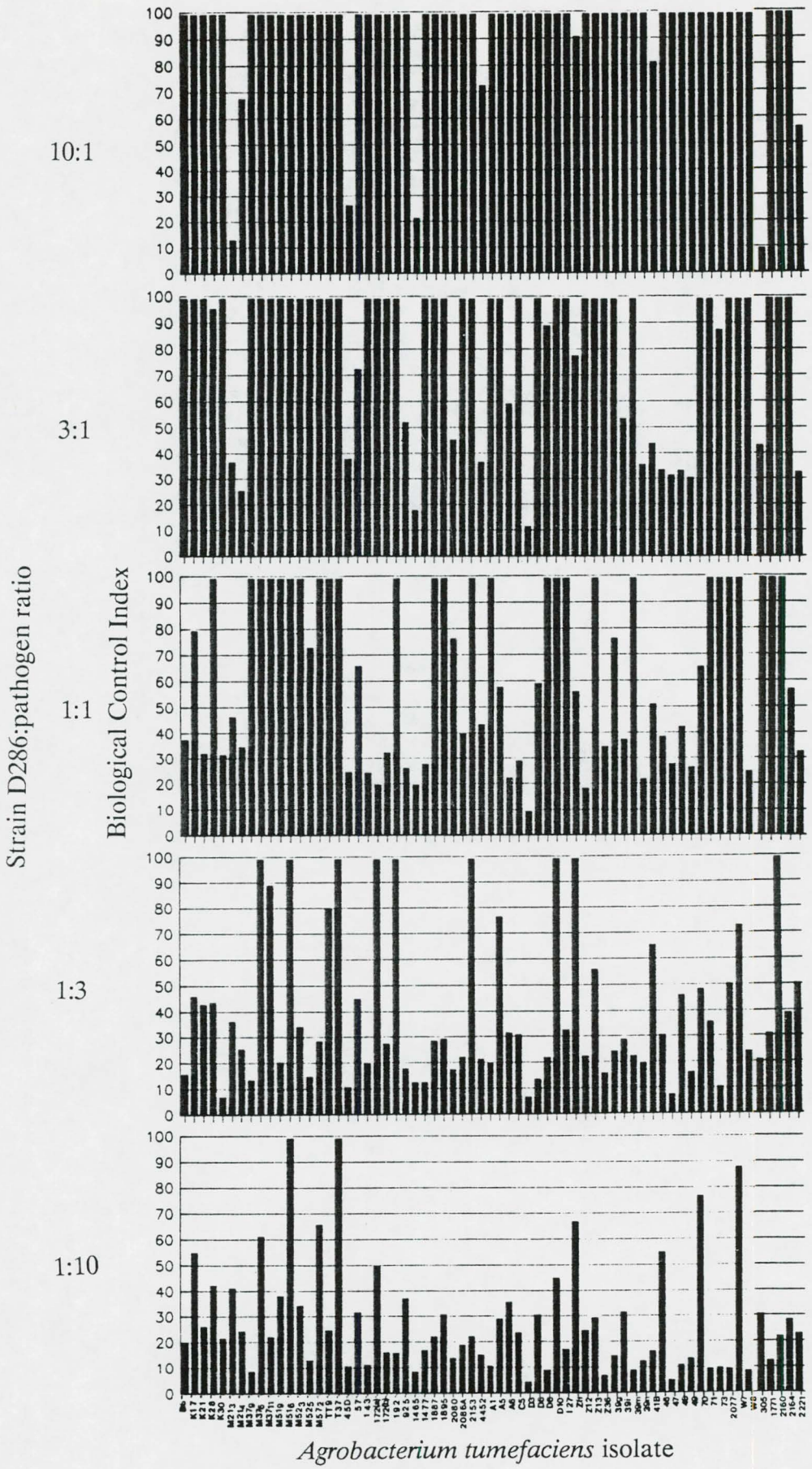


Fig. 31. *In vivo* control of isolates of *A. tumefaciens* by *A. tumefaciens* D286 on tobacco seedlings inoculated with suspensions containing D286 and pathogen cells in different ratios.

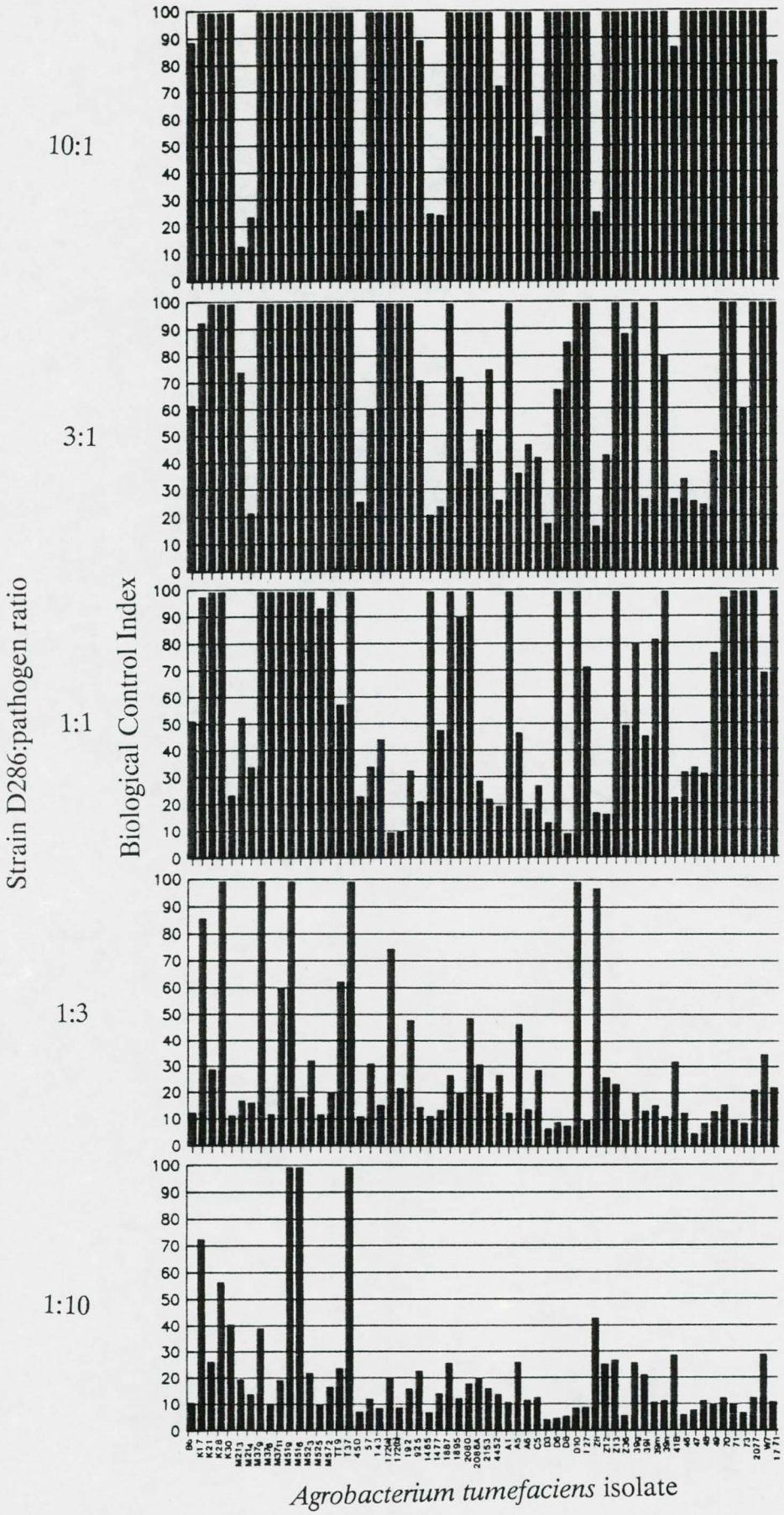


Fig. 32. *In vivo* control of isolates of *A. tumefaciens* by *A. tumefaciens* D286 on datura seedlings inoculated with suspensions containing D286 and pathogen cells in different ratios.

With the application of *A. radiobacter* K84 and the pathogens to tobacco and datura, the control levels decreased as the ratios of control strain to the pathogen decreased (Fig. 29 and 30, Appendix Table 1). The same tendency was observed where *A. tumefaciens* D286 was applied with the pathogens to tobacco and datura (Fig. 31 and 32, Appendix Tables 1 and 2).

The percentages of pathogenic isolates controlled with the different ratios of control strain to isolate are shown in Table 25. It is clear that the percentages of control were higher where *A. radiobacter* K84 was applied than where *A. tumefaciens* D286 was applied. Where *A. tumefaciens* D286 was the control strain, the percentages of pathogenic isolates controlled were mainly higher on tobacco than on datura.

The amplification of tumours also increased as the control strain:pathogen ratio decreased (Appendix Tables 1 and 2).

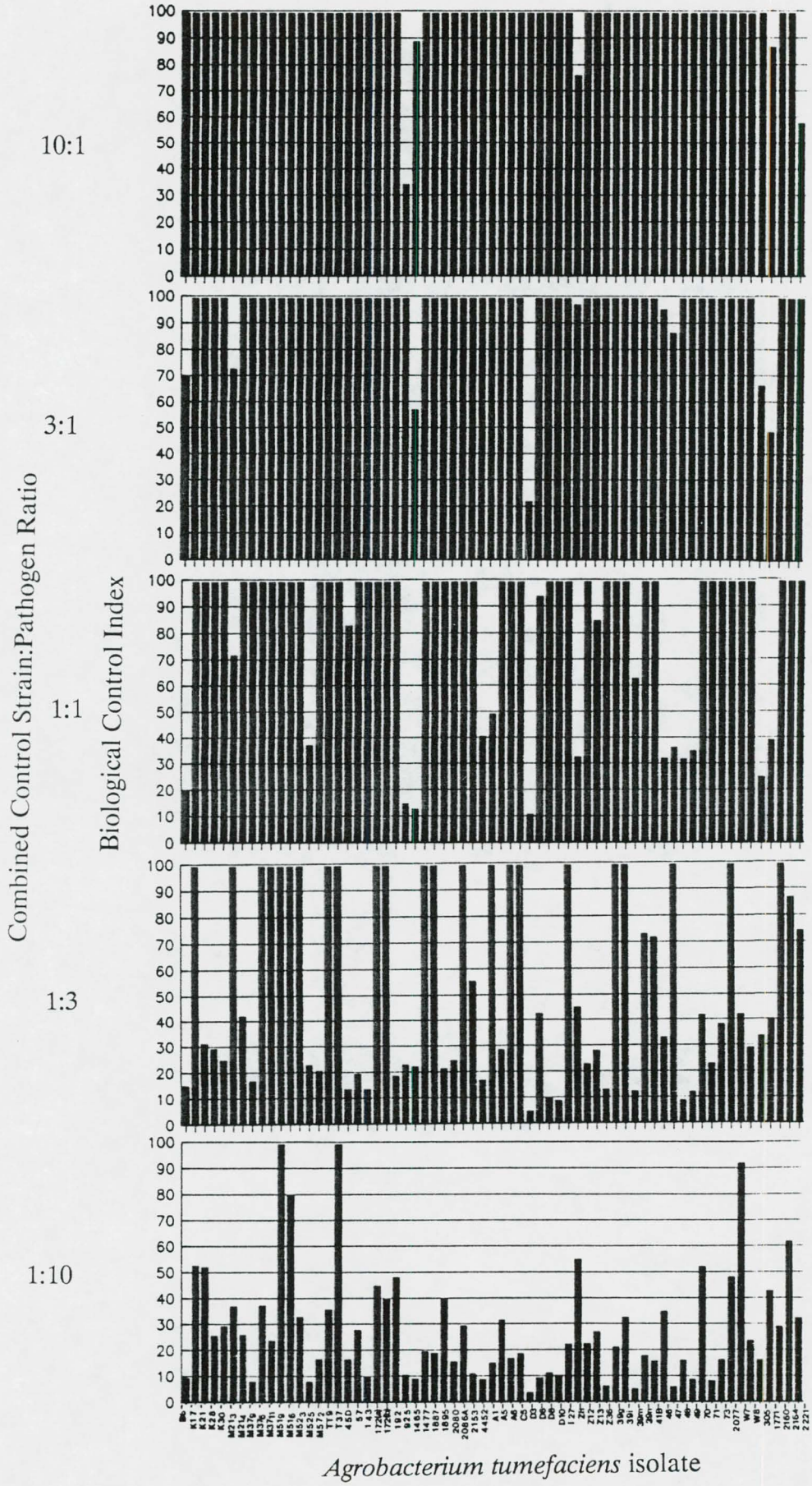
Investigation of possible synergistic action of *Agrobacterium radiobacter* K84 and *Agrobacterium tumefaciens* D286 against different pathogenic isolates

When the combination of both control strains in different ratios was applied to tobacco and datura plants together with pathogenic isolates, the control levels decreased as the ratio of combined control strains to the pathogen decreased (Fig. 33 and 34; Appendix Tables 1 and 2).

The percentages of control by the combined control strains, *A. radiobacter* K84 and *A. tumefaciens* D286, applied with the pathogens in different ratios, are shown in Table 26. Higher percentages of control were achieved on datura than on tobacco at all ratios tested. The combination of control strains (Table 26) produced higher percentages of control when compared to the individual control strains (Table 25), except on tobacco at the lowest control strain:pathogen ratio. (The treatments where the control strains were applied before and after the pathogenic isolates, were also the application of cells in a 1:1 ratio; however, in these time

TABLE 25. Percentages of control with the simultaneous application of pathogenic isolates and bacteriocinogenic strains in different ratios on tobacco and datura plants

Control strain	Test plant	Control strain:pathogen ratio				
		10:1	3:1	1:1	1:3	1:10
K84	Tobacco	90.8	80.0	50.8	32.3	9.2
K84	Datura	96.7	81.5	50.0	23.3	6.7
D286	Tobacco	86.2	64.6	41.5	13.8	3.1
D286	Datura	80.0	48.3	36.7	8.3	5.0



Agrobacterium tumefaciens isolate

Fig. 33. *In vivo* control of isolates of *A. tumefaciens* inoculated onto tobacco seedlings in different ratios with 1:1 mixed suspensions of *A. radiobacter* strain K84 and *A. tumefaciens* D286.

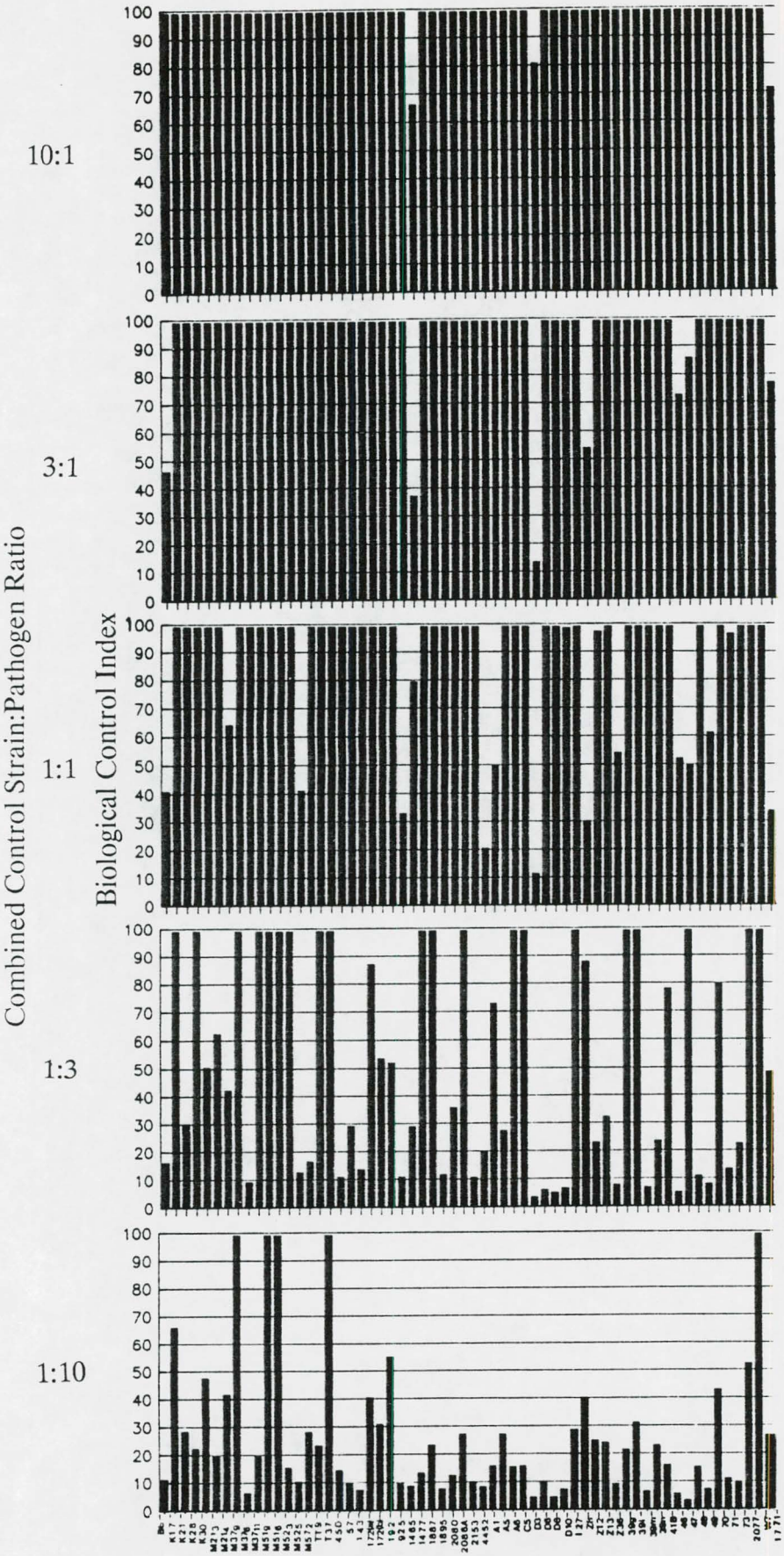


Fig. 34. *In vivo* control of isolates of *A. tumefaciens* inoculated onto datura seedlings in different ratios with 1:1 mixed suspensions of *A. radiobacter* strain K84 and *A. tumefaciens* D286.

TABLE 26. Percentages of control in the combined application of *A. radiobacter* K84 and *A. tumefaciens* D286 control strains to pathogenic isolates in different ratios

Test plant	Combined control strain:pathogen ratio				
	10:1	3:1	1:1	1:3	1:10
Tobacco	92.3	86.2	70.8	35.4	3.1
Datura	95.0	88.3	71.7	33.3	8.3

related studies twice as many control strain and pathogen cells were applied as where the two control strains were combined).

The amplification of tumour development was also observed where the control strains were combined and used in a decreasing ratio compared to the pathogen. As the control strain mixture:pathogen ratio decreased, the incidence of amplification increased on both tobacco and datura plants (Appendix Tables 1 and 2).

On tobacco some isolates ($M21_3$, 4452, 305) were controlled by the combined control strains but not by the separate control strains in the 10:1 ratio of control strain:pathogen. Some isolates (925, 1771) were controlled completely by the separate control strains but not with the combined control strains (Appendix Table 1).

On datura some isolates (4452, Z8) were controlled by the combined control strains but not by the separate control strains in the 10:1 ratio of control strain:pathogen. One isolate (D3) was controlled completely by the separate control strains but not with the combined control strains (Appendix Table 2).

Statistical comparison of *in vivo* control treatments

Comparisons of the host plant responses to the biological control treatments with the agrocinogenic agrobacteria, showed inhibition of tumour formation on tobacco and datura to a greater or lesser extent by almost all the different methods of applying the control strains *A. radiobacter* K84 and *A. tumefaciens* D286 (Tables 27 and 28). The best control (overall mainly >90%) on both host plants was observed where the control strain was applied to wounds 24-30h before the pathogenic strain, or by the simultaneous application of the agrocin producer(s) and pathogen at a ratio of 10:1 or 3:1. Inhibition of tumour formation also tended to decrease progressively as the cell ratio of agrocin producer:pathogen applied to wounds decreased from 10:1 to 3:1 to 1:1. Combinations of *A. radiobacter* K84 and *A. tumefaciens* D286 with pathogen

TABLE 27. Overall effect of method of application of agrocinogenic *A. radiobacter* K84 and *A. tumefaciens* D286 on tumour formation on tobacco by all isolates of *A. tumefaciens* tested

Treatment	Biological control index[a] of tumour formation
No pathogen	99.00 a[b]
K84 added 24 h before pathogen	98.14 a
D286 added 24 h before pathogen	97.05 ab
K84 + D286:pathogen (10:1)	96.15 ab
K84:pathogen (10:1)	94.42 ab
K84 + D286:pathogen (3:1)	94.14 ab
D286:pathogen (10:1)	91.58 ab
K84:pathogen (3:1)	90.32 b
K84 + D286:pathogen (1:1)	81.94 b
D286:pathogen (3:1)	78.54 c
K84:pathogen (1:1)	70.02 d
D286:pathogen (1:1)	63.22 de
K84:pathogen (1:3)	60.19 ef
K84 + D286:pathogen (1:3)	53.80 f
Pathogen added 24 h before D286	41.15 g
D286:pathogen (1:3)	39.98 g
Pathogen added 24 h before K84	37.79 g
K84:pathogen (1:10)	33.90 h
K84 + D286:pathogen (1:10)	28.20 i
D286:pathogen (1:10)	27.81 i
Pathogen only	16.38 j

[a]The closer the index is to 100 the better was the control of tumour formation.
[b]Numbers followed by the same letter(s) did not differ significantly (P = 0.01).

TABLE 28. Overall effect of method of application of agrocinogenic *A. radiobacter* K84 and *A. tumefaciens* D286 on tumour formation on datura by all strain of *A. tumefaciens* tested.

Treatment	Biological control index[a] of tumour formation
No pathogen	99.00 a[b]
K84:pathogen (10:1)	97.69 ab
K84 + D286:pathogen (10:1)	97.59 ab
D286 added 24 h before pathogen	95.35 ab
K84 added 24 h before pathogen	95.01 ab
K84 + D286:pathogen (3:1)	94.25 abc
K84:pathogen (3:1)	93.19 abc
D286:pathogen (10:1)	89.49 bc
K84 + D286:pathogen (1:1)	86.11 c
K84:pathogen (1:1)	74.55 cd
D286:pathogen (3:1)	72.20 d
D286:pathogen (1:1)	63.29 e
K84 + D286:pathogen (1:3)	55.49 e
K84:pathogen (1:3)	55.23 e
K84:pathogen (1:10)	36.19 f
Pathogen added 24 h before D286	35.05 f
D286:pathogen (1:3)	34.62 f
Pathogen added 24 h before K84	32.53 f
K84 + D286:pathogen (1:10)	32.48 f
D286:pathogen (1:10)	27.37 fg
Pathogen only	19.55 g

[a]The closer the index is to 100 the better was the control of tumour formation.
[b]Numbers followed by the same letter(s) did not differ significantly (P = 0.01).

in a 10:1 ratio produced the same high percentage of inhibition (>95%) as the individual strains applied 24-30h before application of the pathogen.

With the exception of *A. tumefaciens* D286 (1:1) on tobacco (Table 27), *A. radiobacter* K84 was more effective than *A. tumefaciens* D286 in inhibiting tumour formation on any of the tobacco or datura plants when applied with the pathogens at cell ratios of 3:1, 1:1, or 1:3, as well as 1:10 on tobacco (Tables 27 and 28). A combined cell suspension of *A. tumefaciens* D286 and *A. radiobacter* K84 was overall just as effective as *A. radiobacter* K84 alone on both tobacco and datura. Exceptions to this result were the 1:1 ratio on tobacco where the combined control strains were more effective, and the 1:10 ratio on tobacco where *A. radiobacter* K84 alone was better. *Agrobacterium tumefaciens* D286 was less effective than the combined *A. tumefaciens* D286 and *A. radiobacter* K84 suspension on both tobacco and datura when applied at ratios of 3:1, 1:1 and 1:3.

Comparison between *in vitro* inhibition and *in vivo* control treatments

The percentages of treatments where tobacco and datura produced no tumours after the application of the control strains, compared to the *in vitro* sensitivity of the pathogenic isolates to the agrocins, are shown in Table 29. With the 1:1 ratio of control strain to pathogen, 50% of the isolates which were sensitive or resistant to agrocin 84 *in vitro* produced tumours on tobacco and datura. With the 10:1 ratio, 100% of the strains sensitive *in vitro* were controlled by *A. radiobacter* K84, whereas 90% of the resistant pathogens were also controlled.

With *A. tumefaciens* D286 in the 1:1 ratio to pathogen, only 46.6% and 33.3% of the *in vitro* sensitive strains were controlled on tobacco and datura, respectively; likewise, 38.4% and 34.6% of the *in vitro* resistant pathogens were controlled on tobacco and datura, respectively. With the 10:1 ratio of control strains K84 and D286:pathogen on tobacco and datura more then 80% of the *in vitro* sensitive and resistant isolates were controlled (Table 29).

TABLE 29. Percentage of treatments where plants had no discernable tumours after concomitant inoculation with *A. radiobacter* K84 or *A. tumefaciens* D286 and each of 56 pathogens[a] which were either susceptible or resistant to the agrocin producer *in vitro*.

Agrocin producer	Number of pathogens tested	Reaction *in vitro*	Percentage of pathogens controlled with three ratios of agrocin producer:pathogen on					
			tobacco			datura		
			1:1	3:1	10:1	1:1	3:1	10:1
K84	36	S[b]	50.0	86.1	100.0	50.0	86.1	100.0
	20	R[c]	50.0	85.0	90.0	50.0	90.0	90.0
D286	30	S	46.6	73.3	86.7	33.3	66.6	86.7
	26	R	38.4	61.5	82.2	34.6	46.2	84.6

[a]31 biotype 1 strains and 25 biotype 2 strains.
[b]Susceptible.
[c]Resistant.

D-Glucosamine

In vitro **sensitivity of pathogenic agrobacteria to D-glucosamine.** None of the bacteria tested showed any inhibition zones with D-glucosamine incorporated into the medium.

Treatment of tumours on tobacco and datura indicator plants with D-glucosamine. None of the tumours formed on tobacco and datura plants were controlled (their development inhibited) or destroyed by the daily application of a 0.1M D-glucosamine solution.

Extract of the alkaloid-producing weed *Hypericum perforatum*

In vitro **sensitivity of pathogenic agrobacteria to the** *Hypericum perforatum* **extract.** None of the *A. tumefaciens* pathogens tested was sensitive to the *H. perforatum* solution (no inhibition zones were formed where the solution was incorporated in the medium).

Treatment of tumours on indicator plants with *Hypericum perforatum* **extract.** No control of tumour development was obtained with any of the *A. tumefaciens* treatments after daily application of the *H. perforatum* solution to the tumours for a period of 30 days.

Gallex

In vitro **sensitivity of pathogenic agrobacteria to Gallex.** None of the bacterial strains tested for sensitivity to Gallex *in vitro* showed any signs of sensitivity towards this chemical.

Treatment of tumours on indicator plants with Gallex. A little regression of tumour development, indicated by a decrease in size, was observed after the tumours were treated with Gallex; however, this appeared to result from phytotoxicity of the Gallex, as after a while the

healthy tissue also showed the same shrinking pattern as the tumour tissue. The Gallex-treated tumours did not die or disappear or fall off during the observation period of 30 days.

Gallex was applied to wounds where the tumours were removed, tumorous tissue reappeared after two weeks.

Heat-Sensitivity of *Agrobacterium tumefaciens* Isolates and Host Plant (Peach) Seedlings

Heat-sensitivity of *Agrobacterium tumefaciens* isolates

All isolates subjected to the different heat-treatments produced bacterial growth on all NA plates after incubation at 25°C for 48h. It therefore appeared that even the harshest heat-treatment (60°C for 80 min) failed to eradicate the *A. tumefaciens* pathogens. All heated bacterial suspensions, even the ones that underwent the harshest treatment, were still able to produce tumours on tobacco seedlings.

Heat treatment of peach (*Prunus persica* cv. Kakamas) seedlings

The peach seedlings did not respond well to the heat treatments of 30 to 60°C for 15 minutes, as all the treated seedlings were overall in a poorer condition than the untreated controls.

DISCUSSION

Isolation of *Agrobacterium tumefaciens* from Diseased Plant Material

Pathogenic *A. tumefaciens* cultures were isolated from crown galls on various host plants from all over South Africa. The widespread occurrence of the pathogen is indicated by the work of Du Plessis *et al.*, (1984), Loubser (1978) and Matthee *et al.* (1979). Crown gall was detected countrywide in commercial nurseries, especially in and around big cities, such as Cape Town, Durban, Johannesburg and Pretoria. It was mainly *A. tumefaciens* biotype 1 isolates that appeared to be the problem (in nurseries) on marguerites (*Chrysanthemum* sp.), of the Double Yellow variety. The biotype 2 pathogens usually infected ornamental and stone-fruit trees, but they were reasonably well controlled by the use of the antagonistic biological control strain *A. radiobacter* K84. No biotype 3 isolates have so far been isolated from commercial nurseries.

In stonefruit nurseries, biotype 2 *A. tumefaciens* had become as a big threat to the industry. The pathogen was devastating, as was also reported by Schroth *et al.* (1971) for stonefruits and roses in Australia and for apples and pears in the USA. The main cause of the problem was that the roots of the plants were usually affected, causing the plants to be dwarfed and predisposed to other more devastating diseases. So far no serious crown gall infections have been reported from South African rose and pome- fruit nurseries.

Economic losses in the USA have resulted in laws prohibiting the interstate shipments of diseased plants and ordering the destruction of such plants (Moore and Warren, 1979). Such a law also exists in South Africa and the sale of infected plants is prohibited under the Agricultural Pest Act (Act 36 of 1983). Infected plants are usually destroyed by the nurserymen. Since 1979, nearly half a million plants have been destroyed following inspections by plant inspectors in the service of the Directorate of Plant and Liquor Control; this figure excludes the plants destroyed by the nurserymen prior to inspections and also excludes the number of infected plants destroyed by farmers. The cost factor involved in South Africa is unknown, but Schroth *et al.* (1971) stated that the annual losses in the State of California alone,

exceeded seven million dollars in one year. The biggest danger, as was also found in Australia by Kerr (1969a), was the selling of plants free of visual symptoms but which could have been latently infected or could have harboured the pathogen in soil surrounding the roots.

The biggest problem at the present time appears to be crown gall on grapevines, caused by *A. tumefaciens* biotype 3 isolates. This disease can destroy grapevines (Erasmus *et al.*, 1974; Loubser, 1978), especially when the plants are still young (Moore and Tingey, 1976). Local biotype 3 isolates have mainly been isolated from the crown areas and on the graft unions; however, Panagopoulos *et al.* (1978) isolated biotype 3 bacteria mainly from the aerial parts of grapevines. In other parts of the world (Panagopoulos *et al.*, 1983), as well as in South Africa, biotype 3 pathogens have so far been isolated only from grapevines and on a limited scale from chrysanthemums in Italy (Bazzi and Mazzucchi, 1978). Very few crown gall infections caused by biotype 3 *A. tumefaciens* are normally found in vineyards in the Western Cape region, although this biotype can be abundant in certain viticultural areas, such as in grapevine nurseries in the Wellington area. Crown gall is abundant in vineyards along the Olifants and Orange River areas in the Western, North-western and Northern Cape, as well as in the Waterberg area in the Transvaal. It appears that rootstock cultivars such as Jacquez, 99 Richter and 110 Richter are more prone to crown gall infection than other rootstocks. The search will continue for rootstock material resistant to the crown gall pathogen.

The crown gall disease pattern may fluctuate from year to year, as was also recorded by Kerr (1969a) and Moore and Warren (1979). Furthermore, it has been experienced in the local nursery industry that grapevines infected with crown gall bacteria showed no disease development after being planted out in Western Cape vineyards, but extensive tumour development when they were planted along the Olifants or Orange Rivers, as well as in the Waterberg area. A possible reason for this phenomenon might be that some environmental factor in the Western Cape prevents the *A. tumefaciens* T-DNA from being expressed with the resultant formation of tumour outgrowths. The expression appears to be completely halted, although it is known that the T-DNA was transferred to the plant cells during infection at nursery level as was found by this laboratory in subsequent studies. This possible

environmental factor could be the climatic conditions existing in the Western Cape in contrast to the different conditions in other regions. The Western Cape is a winter rainfall region usually with cold, wet winters during which plant material is dormant. The other areas mentioned lie within the summer rainfall belt (or are flood irrigated from rivers) and usually have wet, hot summers and cold, dry winters. In these areas, the intensely warm and wet summer conditions, when the plant material is highly active, might predispose the plants for infection by *A. tumefaciens* through wounds. The different soil types, latitude and height above sea level may also play a role in the expression of the crown gall disease.

Large parts of the Cape Province are virtually devoid of crown gall infections as these regions are mainly sheep-farming areas. Large areas of the Orange Free State, Natal and Transvaal also have little crown gall because of the cultivation of monocotyledonous crops like maize, wheat and sugarcane. *Agrobacterium tumefaciens* does not form tumours on monocotyledonous plants (Lippincott and Lippincott, 1977), possibly because the galacturonic acid residues of monocotyledonous plants are esterified to such an extent that binding of *A. tumefaciens* is impossible (Sequeira, 1978).

In the present study, young tumorous tissue yielded the highest number of pathogenic isolates, as was also found by Moore *et al.* (1980). However, some fresh tumour material failed to yield the pathogen, as was also found by Alconero (1980), Gram and Weber (1953), Kerr (1969b) and C. G. Panagopoulos (Benaki Phytopathological Institute, Athens, Greece, personal communication). A reasonable explanation for this phenomenon could be that the tumour tissue mother cells contain the T-DNA insertion sequence which invokes the production of new tumorous tissue in the new growing season in the absence of the causative bacterium, as *A. tumefaciens* itself is no longer required after the infection process (Braun, 1959; Kado, 1976; Veldstra, 1972). Such results suggest that the presence of *A. tumefaciens* in galls could vary considerably. Sometimes the tumour tissue becomes partially organized, producing outgrowths of differentiated cell stuctures called teratomas (Fig. 9E).

Isolations from old and woody tumours were usually unsuccessful, as was also experienced by Gram and Weber (1953) and Kiraly *et al.* (1970). In some cases isolations were made successfully, but they always produced very low yields of *A. tumefaciens*.

The best medium for isolating *A. tumefaciens* from field specimens was NA. On NA *Agrobacterium* colonies were easily identified and selected by their appearance as whitish, circular and convex colonies of 2-4 mm diameter. The main advantage of this medium was its non-selective properties toward *Agrobacterium* isolates. Relatively little contamination by other bacteria and fungi was encountered, except in some cases where a fluorescent, non-pathogenic *Pseudomonas* sp. was identified in tumour material together with *Agrobacterium tumefaciens*. The selective media of Schroth *et al.* (1965) (biotype 1), New and Kerr (1971) (biotype 2) and Brisbane and Kerr (1983) (biotype 3) did not always yield a pathogen which was obtained on NA, probably because of the small numbers of bacteria present in the tumours or because these bacteria had nutritional requirements not supplied by the selective media.

The grafting of pieces of fresh tumour material from field specimens directly onto suitable indicator plants appeared to be the best method of isolating the causative organism, as was also found by Panagopoulos and Psallidas (1973). This procedure also demonstrated that the pathogen was present in tumour material, without involving the time-consuming procedure of isolation and purification of the bacteria, followed by their inoculation onto a suitable indicator plant. Compared with this latter procedure, the direct grafting of tumour material can save up to 2 weeks in the detection of the pathogen. The pathogenic agrobacteria isolated subsequently from the tumours on the indicator plants nearly always appeared as almost pure cultures on the NA plates. However, even the grafting process does not always obtain the pathogen from a diseased plant; the bacteria are not necessarily present in the specimen tumours, as only their T-DNA is required within the tumour cells to make the tumour tissue self-sustaining following elimination of all cells of the causal organism (Stapp, 1961). This T-DNA gets incorporated into the plant cell DNA and can be expressed repeatedly year after year to maintain tumours on perennial plants.

Many *Agrobacterium*-like colonies isolated from host plant tumours were non-pathogenic, as was also found by Kerr and Panagopoulos (1977). A possible explanation could be that some pathogens, at the infection stage, transferred their T-DNA to the host plant cells, thereby rendering them and their progeny devoid of the tumour-inducing principle and thus non-pathogenic. Another explanation could be that infections occurred on the plant crowns at soil level with tumours being formed by pathogens, but that non-pathogens were also attracted to the wound areas, mainly because of the opines, and were trapped in the fast-developing tumour tissue. It also appeared, in examinations and tests, that the pathogen cells were more abundant in the outer layers of the tumours, as was also found by Gram and Weber (1953) and Stapp (1961). Pathogenic bacteria were rarely isolated from the deeper layers of tumour tissue, as was also experienced by C.G. Panagopoulos (Benaki Phytopathological Research Institute, Athens, Greece, personal communication).

Isolation of *Agrobacterium tumefaciens* from Soils

Isolations from soils using non-selective media like NA and YMA were problematical as large populations of fast growing bacteria which were resident in the soils overgrew and completely masked the slower growing *Agrobacterium* colonies. This problem was also experienced by Chilton *et al.* (1976), Kerr (1969a) and Schroth *et al.* (1965). The problem was evident even at high dilutions of soil samples. However, the media of Schroth *et al.* (1965) and New and Kerr (1971) proved to be highly selective for *Agrobacterium* biotype 1 and 2 isolates, respectively, and permitted their successful isolation from soil. The value of the two selective media was also established by Miller and Vruggink (1981), Moore *et al.* (1980), Panagopoulos *et al.* (1979) and Schroth *et al.* (1971). No biotype 1 isolates grew on the medium of New and Kerr (1971), as was also found by Sonoki *et al.* (1978). However, Kerr (1969a) found the medium of Schroth *et al.* (1965) unsuitable for the isolation of biotype 1 organisms and Loubser (1978) experienced a lack of success with both selective media. Apparently, according to Schroth *et al.* (1971), it is necessary to have a high *Agrobacterium* population in the soil for a successful isolation of the pathogen on the medium of Schroth *et al.* (1965). This is possibly because the

relatively large quantities of antibiotics in this medium eliminate lower populations of *Agrobacterium*. Moore (1979), however, stated that these selective media were effective for isolating *A. tumefaciens* from soils diluted to a concentration of about 1 x 10^{-3} g/ml, and that higher concentrations lessened the effectiveness of the media. Schroth *et al.* (1965) showed that none of the selective or non-selective media distinguished between pathogenic and non-pathogenic agrobacteria. The selective medium of Brisbane and Kerr (1983) proved to be very useful for the isolation of biotype 3 agrobacteria from both soils and tumours, with relatively pure biotype 3 isolates being obtained from the plants.

Higher populations of *A. tumefaciens* isolates of all three biotypes were found in soils in close proximity to crown gall-infested plants than in soil away from the plants. The latter soil yielded few or no pathogenic isolates; most agrobacteria found in these soils were non-pathogenic. Also, higher populations of *Agrobacterium* strains were found in sandy soils than in clay soils.

Biotyping of *Agrobacterium* Isolates and Strains

All the *Agrobacterium* isolates and strains could be grouped into the traditional three biotypes, two of which corresponded to biotypes 1 and 2 of Keane *et al.* (1970), Kerr and Panagopoulos (1977) and Panagopoulos and Psallidas (1973), groups I and III of White (1972), clusters 1 and 2 of Kersters *et al.* (1973), the species *A. tumefaciens* and *A. rhizogenes* as described by Holmes and Roberts (1981) and clusters 3 and 1 of Du Plessis *et al.* (1984). The third biotype corresponded to the biotype 3 described by Kerr and Panagopoulos (1977), the species *A. rubi* as described by Holmes and Roberts (1981) and cluster 2 of Du Plessis *et al.* (1984). The majority of isolates in the present study (35) resorted under biotype 1 and the fewest (8) under biotype 3. The reason why so few biotype 3 isolates were obtained was perhaps because of their limited host range (grapevines).

The biotyping tests as described by Kerr and Panagopoulos (1977) were easy to perform and stable characteristics were shown by the bacteria of the different biotypes. Properties common to all biotypes were a negative Gram reaction, motility, no fluorescent pigment production on

King's medium B, a positive oxidase reaction and the oxidative utilization of glucose. Some variation was observed among isolates placed in a particular biotype in respect of the rest of the tests, as was also found by Kerr and Panagopoulos (1977). The greatest variation was observed within the biotype 3 group. The variation within biotypes is not surprising as Du Plessis *et al.* (1984) found several distinct genetic groups within each of their three clusters which corresponded to the biotypes 1, 2 and 3.

Some isolates (M27, 1887) of the biotype 1 group produced no 3-ketolactose, although Keane *et al.* (1970) and Kerr (1969a) found all biotype 1 isolates to produce 3-ketolactose. Most biotype 2 isolates produced similar results to the biotype 2 isolates described by Du Plessis (1983) and Kerr and Panagopoulos (1977). However, isolates 1671 and 2077 failed to utilize citrate as a sole carbon source. Biotype 2 isolates D3, D6, D7, D8 and D10 were unusual as they grew at 37°C and did not utilize malonate and tartrate. Du Plessis (1983) found that isolates D7, D8 and D10 grew at 35°C but not at 37°C, and also that isolates D7, D8, D9 and D10 were closely related to the biotype 1-2 of Spiers (1979). However, in the present study, unlike the biotype 1-2 isolates of Spiers (1979), isolates D7-10 did not produce 3-ketolactose, nor an alkaline reaction in litmus milk or on malonate. They all produced acid from erythritol, as was also found by Du Plessis (1983), thus linking them to the biotype 1-2 group of Spiers (1979). The few variations revealed by these isolates were not enough to separate them into a different biotype. Isolates 70 and 71, which were found in the present study to belong to the biotype 2 group as described by Kerr and Panagopoulos (1977), were considered by Du Plessis (1983) to be atypical biotype 3 isolates. However, the numerical analysis study of Du Plessis (1983) of the 47 phenotypic characters showed isolates 70 and 71 to be phenotypically more related to the biotype 2 group. It is doubtful whether these isolates, obtained from *Prunus* sp., could be atypical biotype 3 isolates, as typical or atypical biotype 3 isolates have so far been obtained only from grapevines and *Chrysanthemum* sp, not from stonefruit trees, as was also found by Loubser (1978), Staphorst *et al.* (1985) and Süle (1978).

Among the biotype 3 isolates, five physiologically different groups were distinguished (Table 2). Biotype 3 isolate 305 was the only one to produce reactions similar to those described by

Kerr and Panagopoulos (1977). As few biotype 3 isolates have been studied so far, it is possible that the biotype 3 strains in South Africa are a rather heterogeneous population. Isolates 1771, 2160, 2164, 2221, W7 and W8 differed from the biotype 3 described by Kerr and Panagopoulos (1977) in that they grew at 37°C and did not utilize citrate. Furthermore, 1771, 2164, W7 and W8 utilized propionate as well as malonate and tartrate. These results are also not in complete agreement with those of Du Plessis (1983), who recorded that strain 1771 did not grow at 37°C or 35°C, nor utilized tartrate. However, the phenotypic characteristics of these biotype 3 strains and isolates did not differ so drastically from the biotype 3 descriptions of Kerr and Panagopoulos (1977) as to put them in a different biotype.

None of the biotype 2 and 3 isolates produced acid from melezitose, in contrast to some of those tested by Miller and Vruggink (1981). No distinctions could be made in the present study between biotype 1 and 2 isolates on the basis of the oxidase reaction, in contrast with the findings of Dhanvantari (1976). All of the biotype 1 isolates in the present study grew on the biotype 1 selective medium of Schroth *et al.* (1965), and all the biotype 2 isolates grew on the biotype 2 selective medium of New and Kerr (1971). However, Du Plessis (1983) reported that the biotype 1 isolates B6, 143 and 4452 failed to grow on the Schroth *et al.* (1965) medium. In other studies conducted by Miller and Vruggink (1981) the selectivity of the media was doubtful as all the biotype 1 isolates tested grew on the medium of New and Kerr (1971) and a third of the biotype 2 isolates tested grew on the medium of Schroth *et al.* (1965).

All *A. tumefaciens* isolates found on *Chrysanthemum* sp. belonged to the biotype 1 group. Similar results were obtained by Miller (1975) in New Zealand and in the USA, but Bazzi and Rosciglione (1982) subsequently reported the isolation of biotype 3 *A. tumefaciens* from a *Chrysanthemum* sp. in Italy. However, their results do not prove that these isolates really belonged to the biotype 3 group as they displayed differences from the biotype 3 isolates of the present study and those of Du Plessis (1983) and Kerr and Panagopoulos (1977). It appears from these three studies and those of Loubser (1978), Staphorst *et al.* (1985) and Süle (1978) that biotype 3 strains may have a host range restricted to grapevines only. Biotypes 1 and 2 are

not restricted in this way to a single host genus (Kerr and Panagopoulos, 1977; New and Kerr, 1972; Süle, 1978).

All isolations from stonefruits, like those of Panagopoulos *et al.* (1979), consisted of biotype 1 and 2 strains. Results from the present study indicate that the biotype 1 isolates were the most abundant, unlike the results obtained by López (1978), New and Kerr (1971), Panagopoulos and Psallidas (1973) and Süle (1978), where the biotype 2 isolates were the most abundant. Some correlations were found between some of the biotypes and geographical areas, whereas Süle (1978) appeared to have found none.

The biotype tests could not distinguish between pathogens and non-pathogens, as was also reported by Abo-El-Dahab *et al.* (1978), Du Plessis (1983), Du Plessis *et al.* (1984), Moore *et al.* (1980) and Schroth *et al.* (1971). There was relatively little variation of characteristics within biotypes 1 and 2 according to the biotype tests, although the isolates were obtained from different hosts and different geographical areas. Little variablilty among strains within the two biotypes was also found by Keane *et al.* (1970) and Panagopoulos and Psallidas (1973).

Standardization of *Agrobacterium* Cell Suspensions

Colorimetric measurement of the absorbance of cell suspensions provided an easy and rapid method for their standardization (adjustment to known cell concentrations) for serological studies or applications of pathogens to wounds without or together with non-pathogens. A standard graph was constructed for this purpose from simultaneous absorbance determinations and dilution plate counts on cell suspensions of two representative isolates. After trials with blue and green filters, it was decided to use only the green filter readings because of the higher r^2 values for the absorbance-dilution plate count regression, and also to use the standard graph only to a cell reading of 3×10^9 cells/ml. The regression for higher cell concentrations showed too much deviation to be of use for standardizing bacterial cell suspensions.

Evaluation of Indicator Plants for *Agrobacterium tumefaciens* Biotype 1, 2 and 3 Isolates

Of the plants tested as hosts for *A. tumefaciens* biotype 1 and 2 isolates, tobacco and datura seedlings were the best indicators of tumour formation. Their value was confirmed in the experiments on the *in vivo* control of tumour formation by the bacteriocinogenic *Agrobacterium* K84 and D286 strains (Appendix Tables 1 and 2). Tobacco (Anderson and Moore, 1979; Keane *et al.*, 1970; Kerr, 1969a; Kerr and Panagopoulos, 1977) and datura (Anderson and Moore, 1979; Moore *et al.*, 1980; Panagopoulos and Psallidas, 1973; Schroth *et al.*, 1965) are widely used as indicator plants. The plants are very easy to cultivate, have high germination percentages and are usually very uniform in their growth, making them highly suitable for experimentation. They are also not very susceptible to other diseases. Tumours on these plants were usually three to six times larger than the tumours on the broadbean and tomato plants. Tobacco and datura seedlings proved to be non-selective towards all *A. tumefaciens* biotype 1 and 2 isolates tested.

All biotype 3 isolates and strains produced tumours on tobacco (Appendix Table 1) but only two (W7, 1771) produced tumours on datura plants (Appendix Table 2). The other *A. tumefaciens* biotype 3 isolates (W8, 305, 2160, 2164, 2221) produced no tumours on datura. On tobacco the tumours produced by *A. tumefaciens* biotype 3 isolates varied in size, with some tumours, for example, those induced by isolate W7, being very small. This could possibly be because the biotype 3 isolates appear to have a host range restricted outside the laboratory to grapevines, and are adapted to this host in respect of the induction of large tumours.

The broadbean and tomato plants were found to be unsuitable due to low germination percentages, non-uniformity and secondary infections probably caused by seed-transmitted pathogens. Broadbean and tomato also resisted infection by certain biotype 1 and 2 isolates. Sunflower seedlings were also found to be suitable for the biotype 1 and 2 isolates, but tended to occupy too much bench space in the environmental growth chambers and glasshouses. Panagopoulos and Psallidas (1973) and Süle (1978) found that *A. tumefaciens* biotype 3 isolates

produced tumours on sunflower seedlings. In the present study none of the sunflower seedlings produced any tumours when inoculated with the eight biotype 3 isolates.

Carrot discs were also found to be unsuitable due to the long periods needed for tumour expression, as well as their susceptibility to soft-rotting by bacterial contaminants, as was also experienced by Schroth *et al.* (1971).

Because of the poor tumour production (qualitatively or quantitatively) on tobacco and datura plants by biotype 3 isolates, different vegetatively propagated grapevine rootstocks were tested for susceptibility to the biotype 3 isolate 2221. It appeared that Hanepoot, Jacquez and Sultana were the most susceptible to this biotype 3 pathogen. Further studies concentrated on the susceptibility of the Jacquez grapevine cultivar as an indicator host, as this is the cultivar most affected by *A. tumefaciens* infections, both in nurseries and in vineyards. In this connection, the Jacquez rootstock is highly prone to mechanical damage by different cultivation practices.

As the propagation of vegetative rootstock material is so time-consuming, it was decided to use Jacquez seedlings in susceptibility trials with the eight *A. tumefaciens* biotype 3 isolates. The germination was satisfactory and the subsequent propagation of the material proved to be much easier and required much less space than the propagation of vegetative material. The Jacquez seedlings were highly susceptible as they always produced tumours when inoculated with any pathogenic biotype 3 isolates. Tumours on these seedlings could be noticed after only 12 days of incubation. The biggest advantage of the Jacquez seedlings was probably that they could be produced throughout the year and were not bound to season like the vegetatively propagated material. The use of Jacquez seedlings has since become standard practice in various laboratories in South Africa for pathogenicity tests of apparent *A. tumefaciens* biotype 3 isolates.

Serological Studies

The serological studies were undertaken mainly as attempts to differentiate serologically between biotype 1 and 2 isolates, as was achieved by Keane *et al.* (1970). Other aspects investigated were correlations of serological characteristics with host specificity, geographical distribution, and sensitivity to agrocins, as well as serological differences between bacteria containing a polysaccharide outer layer and bacteria devoid of such an outer layer.

Plates of YMA appeared suitable to cultivate bacteria producing a polysaccharide outer layer and NA plates to cultivate bacteria with very little slime (polysaccharide) production.

In the initial antigen-antiserum agglutination tests, it appeared that the best procedure was that where the test tubes were incubated in a waterbath at 47°C for 4-5 h and the final reaction recorded after overnight storage at 4°C. Reactions were very clear and sharply defined in comparison with the more conventional method of incubating test tubes at 37°C for 3 h.

In the initial Ouchterlony immunodiffusion tests with the various treatments of both cell types, the phenol treatment appeared to produce the best results, giving better, easier and quicker extraction of diffusible antigens than the other methods, as was previously found by Vruggink and Geesteranus (1975). The untreated cells produced diffuse precipitin bands, probably because of the failure of the reacting antigenic determinants to diffuse through the plates at a constant rate because they were bound either to cell refuse or to other chemicals diffusing at different speeds. The heat-treated cells produced fine and well defined precipitin bands, although not as sharp as those of the phenoltreated cells; however, some isolates showed precipitin bands with the untreated and phenol-treated cells but not with the heat-treated cells. This could probably be because the heat caused the dissociation of the antigens into non-antigenic subunits as was found with *Erwinia carotovora* (Vruggink and Geesteranus, 1975). It was also proven, by using the supernatant liquid of bacterial cell cultures, that some antigens were secreted into the surrounding medium. These antigens were probably pieces of envelope material, possibly polysaccharide, and produced diffuse precipitin bands. The ultrasonically

treated cells also showed weak and diffuse precipitin bands. All the bands formed with ultrasonically treated cells were single as opposed to multiple precipitin bands found by Hochster and Cole (1967), Graham (1971) and Roberts and Kerr (1974),. It therefore appears that the ultrasonic treatment in this study either did not effectively solubilize the antigens of the cell envelope or caused the antigens to be partly destroyed in the process. By contrast, the phenol treatments seemed to liberate the antigens from the cells surfaces effectively, enabling them to diffuse into the surrounding medium.

With the Wasserman tube agglutination method, different serogroupings were obtained with type A (minimally washed) cells and type B (well washed) cells; however, the B-type antigens were tested against more antisera than the A-type antigens. The type A antigens (cells) could be grouped on the basis of their O-reactions with three antisera into seven different serogroups (Table 10), with serogroups I, VI and VII containing the largest number of *Agrobacterium* isolates. Group I produced an O-antigenic reaction only with antiserum against isolate TT9, group VI with antisera against isolates D3 and 2077 and group VII only with antiserum against isolate 2077. Group V produced a weak O-antigenic reaction with antiserum against isolate D3 only. Groups II to IV and VI produced O-antigenic reactions with antisera against two or more isolates. The A-type cells also showed four groups with different H-antigenic reactions. A large number of isolates produced no H-antigenic reactions, either because these isolates had no H-antigens or had flagellar antigens which did not react with the test antisera.

From the agglutination reactions of type B (well washed) cell preparations with antisera against seven isolates, 18 different serogroups could be distinguished (Table 11). The largest group was group XVI with 10 isolates. The reason why more serogroups were distinguished with the type B antigens was partly because they were tested against more antisera than the type A antigens. However, if the reactions with the antisera used against the type A are considered, seven different serogroups can also be distinguished although showing differences from the grouping for the A-type antigens. No H-antigenic reactions were observed with the type B antigens, showing that the flagellar antigens were all removed by the rigorous washing process.

Examples of isolates for which type A cells showed different reactions from type B cells with antisera against the same isolates, are the biotype 1 isolates 198, 2080 and 2086A (Table 10 and 11). Their type A cells reacted with antiserum against type A cells of TT9 but their type B cells showed no reactions with antiserum against TT9 type B cells. Type A cell preparations of isolate D10 produced an O-antigenic reaction with antisera against type A cells of D3, TT9 and 2077, whereas the type B preparations produced O-antigenic reactions only with the antisera against type B cells of TT9 and 2077. Similarly, the type A and type B cell preparations of various other isolates differed in their serological reactions with antisera against type A and type B cells, respectively, of the same isolates. It appears that there were antigenic determinants of the outer polysaccharide of the type A cells that were absent from the type B preparations; it also appears that some antigenic determinants were masked by the polysaccharide layer on type A cells and exposed on removal of this layer. The two types of cells would also have induced the production in the rabbits of different antibodies, which would also have contributed to the different reactions of the two cell types.

Results obtained in the Ouchterlony immunodiffusion tests with type A cells (Table 12) were very similar to the agglutination results (Table 10). Complete identity of some isolates to certain antisera was observed, for example, with homologous isolate TT9 and heterologous isolate T-37 versus TT9 antiserum, homologous isolate D3 and heterologous isolates D6, D7, D8 and D10 versus D3 antiserum, homologous isolate 2077 and heterologous isolates 41B, A1, A6, C5, 46, 47, 48, 49, Z8 and Z12 versus 2077 antiserum. All the other reaction were reactions of partial identity. The immunodiffusion results with type B cells (Table 13) did not correspond well to the agglutination results with these cells (Table 11). Identity patterns which were very similar to those the type A cells were observed, although a few differences were present as can be seen in Table 13, especially regarding reactions with 2077 antiserum which produced two precipitin bands (A and B) in the reactions with some biotype 2 isolates. The antigens responsible for the unique agglutination reactions of A-type cells would have been loosely bound and probably readily diffusible in the agar gells of the Ouchterlony plates, whereas those giving the unique reactions of B-type cells were probably tightly bound to the cell surfaces

preventing them from diffusing into the surrounding medium. Differences in the agglutination and immunodiffusion results may also have resulted from the direct contact between the antigen and antibodies in the liquid medium in the agglutination tests providing greater possibilities of interactions than are possible in immunodiffusion tests, where the antibodies and antigens have to move through a semi-solid medium in order to come into contact with one another.

Type A (minimally washed) or type B (well washed) cell preparations of isolates W7, W8, 305, 1771, 2160, 2164 and 2221 of the biotype 3 group produced no homologous or heterologous precipitin reactions with antisera prepared against type B cells of isolates 305 and 2221. It appeared as if the homologous antisera precipitated the antigens in the antigen wells, which could mean that the biotype 3 isolates had no diffusible antigens which could move into the surrounding medium. All the different antigen-releasing treatments discussed above were used on these cells, but with no success. However, with the more drastic 'Boivin' extraction method thin and clearly defined precipitin bands were formed by isolates 305 and 2221 with their homologous antisera (prepared against type B cells). It would appear that these isolates had antigens which were so tightly bound to the cell walls that they were retained in a non-diffusible state during general antigen-releasing treatments. Humphrey and Vincent (1965) noted that somatic O-antigens forming structural parts of the cell wall may possibly be retained in this way and be available for agglutination but not for diffusion.

From Table 14 it is clear that the isolates could be grouped in larger and fewer groups (serogroups) on the basis of their main surface O (O/S) antigens than on the basis of deeper O (O/D) antigens. The antigenic determinants on the labile cell surface (which was removed by thorough washing with saline) thus seemed to be fewer, or to occur in fewer combinations, than those situated on the deeper more stable part of the cell envelope. The labile surface O-antigenic determinants are probably associated with the outer part of the cell envelope of these bacteria, consisting mainly of protein, lipid and polysaccharide (LPS) as the major groups of chemical substances (Manasse and Corpe, 1967). The cell outer layer with O-antigens may also be a determinant of pathogenicity as in the case of other Gram-negative bacteria (Osborn

et al., 1964). Thus, site binding specificity depends on the LPS of the cell envelope (Whatley *et al.*, 1976) and it appears, as found by Lippincott and Lippincott (1980), that it is especially the external projecting polysaccharide chains that are involved in the attachment of bacteria to wound cells. These may be O-antigens of the agrobacteria, but whether O/S or O/D is not known.

Overall there were 23 groups of *A. tumefaciens* isolates grouped according to their main O-antigenic affinities with the antisera in the agglutination and immunodiffusion tests (Table 15).

Cambra and López (1978) used the indirect ELISA technique in a serological study on *Agrobacterium* and found this method to be eight times more sensitive than the indirect immunofluorescence technique. Serological studies on *Rhizobium* in pure cultures, mixed cultures and root nodules have also been conducted using ELISA techniques (Kishinevsky and Bar-Joseph, 1978; Kishinevsky and Gurfel, 1980; Morley and Jones, 1980). A disadvantage of the *Agrobacterium* study (Cambra and López, 1978) was that the tests were conducted with only a single isolate. The ELISA tests in the present study were an attempt to find a more sensitive method of serodiagnosis and a method whereby a large number of samples could be processed at the same time. The ELISA technique proved less sensitive in showing positive reactions than the agglutination and immunodiffusion techniques, and in several cases (isolates 172(a) and (b), $M51_6$ and $M52_5$) showed different main antigenic affinities to those shown by the other two techniques. Some heterologous reactions produced low absorbance readings (below 0.1) which were ignored as unreliable. The differences between the ELISA and agglutination reactions were unexpected, as both took place in a liquid medium where the antigens would have come into close contact with the antibodies. By contrast, Kishinevsky and Bar-Joseph (1978) found in their *Rhizobium* studies that the two techniques showed basically the same reactions. The reasons for the discrepancies between the ELISA and agglutination reactions in the present study are unknown. The ELISA technique was also not very sensitive in that it had a high threshold concentration of 10^5-10^7 bacterial cells/ml at which bacteria could just be detected. Below the lower value bacteria, although present, could not be

detected. However, this disadvantage might be overcome by a modification to the preparation of the bacterial suspension so that bacterial numbers could be enhanced.

In this study, each biotype contained several serotypes, and certain serotypes were found in two or three biotypes (Table 18). There was thus no sharp serological distinction between biotypes 1 and 2 as was found by Keane *et al.* (1970). Serotyping at present does not have the potential for rapidly and reliably assigning *Agrobacterium* isolates to specific biotypes.

The association of serogroups with specific host plants was only partial and does not appear to provide a rapid method of pathotype (pathovar) distinction. Certain small serogroups could possibly be linked to certain hosts; however, with other serotypes no such relationship was observed and the serotype formed tumours on more than one host. There also appeared to be some relationship between certain serogroups and geographical area, but no such relationships in other cases (Table 18). The overall picture is that some serogroups, especially the smaller ones consisted of isolates from the same biotype, host plant and geographical area, possibly indicating that in certain areas *A. tumefaciens* of a particular serogroup and biotype had established itself as the crown gall pathogen of the particular host. However, other serotypes showed no such correlation, as in the studies of Du Plessis (1983) and Süle (1978) in which they were unable to show correlations between biotype and geographical location. It would be difficult in modern times to maintain geographic separation of different serotypes and biotypes in different regions of a country as there is a constant influx and movement of different plant material all over the country. There are also the wide host ranges of *A. tumefaciens* biotypes 1 and 2 and certain serotypes to take into consideration. Nevertheless, climatic and soil factors may favour a particular strain in certain areas. Biotype 3 may be restricted to grape-producing areas if its host range is limited to grapevines.

In summary, it is evident that *Agrobacterium* strains are highly heterogeneous in respect of serological characteristics as was also observed by Berquist and Elrod (1948) and by Miller and Vruggink (1981). The present serological investigation of South African *Agrobacterium* isolates, supported by the findings of Miller and Vruggink (1981) and Roberts and Kerr (1974),

indicates that the serological approach provides no easy, rapid and reliable procedure for the routine diagnosis of *Agrobacterium* isolates, as neither biotypes nor pathotypes are represented by single or even a few specific serotypes.

In Vitro Inhibition of *Agrobacterium tumefaciens* Isolates by Bacteriocinogenic Strains *Agrobacterium radiobacter* K84 and *Agrobacterium tumefaciens* D286

Considerable differences were observed with both agrocin 84 and agrocin D286 in the numbers of strains inhibited on the different test media (Table 19). Moore and Warren (1979), Scalza *et al.* (1979) and Smith and Hindley (1978) also found in laboratory tests of agrocin 84 sensitivity, that different growth media could influence the susceptibility of pathogenic *Agrobacterium* isolates to the agrocin. Spiers (1980a) found that the production of agrocin 84 by *A. radiobacter* K84 was influenced by the growth medium, incubation time and temperature. Hendson *et al.* (1983) also observed the same effect with agrocin D286. The observations of different sensitivity patterns on different media complicate the assessment of the *in vitro* sensitivity of pathogens to agrocin-producing control strains (non-inhibited isolates might be inhibited if further media were tested).

Surprisingly low percentages of the *A. tumefaciens* isolates tested *in vitro* were sensitive to the agrocins produced by *A. radiobacter* K84 and *A. tumefaciens* D286 (Table 19,23). On SM medium which is employed worldwide for *in vitro* agrocin sensitivity, only 33.8% of all the *A. tumefaciens* isolates tested were found to be sensitive to agrocin 84 and 9.2% to be sensitive to agrocin D286; however, 56.9% and 50.8% were sensitive to agrocin 84 and D286, respectively, on at least one of the three media tested. Of the biotype 1 and 2 isolates, 63% and 68%, respectively, were sensitive to agrocin 84 on at least one of the test media; the corresponding inhibition percentages with agrocin D286 were 94% and 12%. None of the biotype 3 isolates tested showed any inhibition zones with either of the two agrocins *in vitro*. Du Plessis *et al.* (1984) found 51% of South African *A. tumefaciens* isolates, but no biotype 3 isolates (Du Plessis, 1983), to be sensitive to agrocin 84 *in vitro*. *In vitro* studies in other parts of the world indicated that the pathogen populations might be more homogeneous, as in most cases, all or

most of the pathogenic isolates were sensitive to agrocin 84 (Bonzar *et al.*, 1983; Kerr, 1974, López, 1978; Panagopoulos *et al.*, 1979; Spiers, 1980a). However, Bazzi and Mazzucchi (1978) and Moore (1977) reported biotype 2 isolates to be more sensitive to agrocin 84 than biotype 1 isolates, as was found marginally in this study. Kerr and Panagopoulos (1977) also found biotype 3 isolates to be insensitive to agrocin 84 *in vitro*.

Contrary to the results of Hendson *et al.* (1983), it did not appear that agrocin D286 had a broader host range than agrocin 84. However, the agrocin of *A. tumefaciens* D286 inhibited 12 biotype 1 isolates and six biotype 2 isolates not inhibited by the agrocin of *A. radiobacter* K84. On the other hand, the agrocin of *A. radiobacter* K84 inhibited 14 isolates (all biotype 2) not inhibited by the *A. tumefaciens* D286 agrocin. Inhibition zones by agrocin D286 were also generally much smaller than inhibition zones by agrocin 84, indicating a lower sensitivity of the isolates to the D286 agrocin or poorer production of this agrocin on the test plates or slower diffusion of the agrocin through the test plates than in the case of agrocin 84.

Ellis and Kerr (1978) and Kerr and Htay (1974) observed that only isolates harbouring the nopaline-type Ti-plasmid were subject to inhibition by *A. radiobacter* K84. Miller and Vruggink (1981) found biotype 1 isolates harbouring the octopine-type Ti-plasmid to be insensitive to agrocin 84 and also not subject to biological control. However, no such information was available for the agropine-type plasmid also found in some pathogens. At this stage the opine status of all the South African *Agrobacterium* isolates is not known.

Colonies of agrocin-resistant isolates which developed within the inhibition zones were re-isolated and tested for pathogenicity. The same results were obtained as in the studies by Engler *et al.* (1975), Kerr and Htay (1973) and Roberts and Kerr (1974) namely, that isolates which developed inside the inhibition zones were no longer pathogenic. These agrocin-resistant colonies could not have originated from the agrocin-producing strain situated in the middle of the plates as all the cells were killed by chloroform vapour. The more the agrocin was diluted towards the outer edges of the inhibition zones, the greater was the number of resistant colonies that developed.

In Vivo Inhibition of *Agrobacterium tumefaciens* Isolates by Bacteriocinogenic Strains *Agrobacterium radiobacter* K84 and *Agrobacterium tumefaciens* D286

The control of *A. tumefaciens* infections of plants by *A. radiobacter* K84 has been studied extensively over a period of years, but at the start of the present study no information was available of control by the local strain, *A. tumefaciens* D286. This strain, which would previously have been a 'super-pathogen' because it was both pathogenic and produced a bacteriocin, was found by Hendson *et al.* (1983) to have spontaneously lost its pathogenicity. Loss of its pathogenicity changed it to a potentially useful strain for the control of *A. tumefaciens* infections.

Kerr and Htay (1974) and New and Kerr (1972) observed that a 1:1 ratio of control strain to pathogen produced complete control and that higher control strain to pathogen ratios were unneccessary. However, in the present study increases in the percentages of control were achieved as the concentration of control strain:pathogen increased through the ratios 1:10, 1:3, 1:1, 3:1 to 10:1. Some isolates weakly sensitive to agrocin 84 needed a 3:1 or 10:1 ratio of control strain to pathogen for adequate control, while others were not effectively controlled even at an *A. radiobacter* K84:pathogen ratio of 10:1. From these results and those of Alconero (1980), Du Plessis *et al.* (1985), Kerr and Htay (1974), Moore and Warren (1979) and Scalza *et al.* (1979) it appears that *A. radiobacter* K84:pathogen ratios higher than 1:1 are needed to control some crown gall pathogens. On the other hand, some isolates which were highly sensitive to agrocin 84 were effectively controlled at *A. radiobacter* K84:pathogen ratios of 1:3 or 1:10. Similar results were obtained by Du Plessis *et al.* (1985). Some isolates (1465 and 41B on tobacco; 925, 1465, 1477, C5 and 41B on datura), which were sensitive to *A. radiobacter* K84 *in vitro* were not effectively controlled at a pathogen:*A. radiobacter* ratio of 1:10.

The best inhibition on the tobacco and datura test plants was obtained when the agrocin-producing strains were applied 24-30 h before the pathogen and also by the simultaneous

application of agrocin-producing strains and pathogens at a ratio of 10:1 or 3:1. Inhibition of tumour formation decreased progressively as the cell ratio of the agrocin-producing strains to pathogenic isolates decreased from 10:1 to 3:1 to 1:1.

Under natural conditions the pathogen populations would be much lower than the populations used experimentally in the present study (C.G. Panagopoulos, Benaki Phytopathological Institute, Athens, Greece, personal communication). Some pathogens were not inhibited by the agrocin-producing strains *in vitro* but were controlled on host plants as was also experienced by Moore (1977, 1979) and Schroth and Moller (1976). By contrast, Kerr and Htay (1974) and Kerr and Panagopoulos (1977) found that only *A. tumefaciens* isolates sensitive to agrocin 84 *in vitro* on SM were controlled *in vivo*. Some of the *A. tumefaciens* biotype 3 isolates were even controlled under certain conditions, for instance, where the *A. radiobacter* K84 and *A. tumefaciens* D286 control strains were applied 24-30 h before the pathogens at a control strain:pathogen ratio of 10:1. However, Kerr and Panagopoulos (1977) and Panagopoulos *et al.* (1979) found all biotype 3 isolates from grapevines resistant to biological control by *A. radiobacter* K84.

An interesting amplification phenomenon, previously described by Garrett (1979), was observed where the pathogen and control strain were inoculated together, namely, the production of larger tumours than where the pathogen was inoculated alone. The amplification was especially noticeable where the control strains were applied after the pathogenic isolates.

Certain experimental evidence has suggested that *A. radiobacter* K84 controls crown gall infection by the excretion of agrocin 84 (Cooksey and Moore, 1982a; Kerr and Htay, 1974; Moore, 1977, 1979; Moore and Warren, 1979), which attaches to receptor sites on the cell walls of pathogenic agrobacteria, thereby modifying their structure and preventing attachment of the pathogenic bacteria to the plant cell walls (Reeves, 1972). Other results suggest that the biological control functions by *A. tumefaciens* physically blocking the infection sites on the plant cell walls usually occupied by the pathogen (Lippincott and Lippincott, 1980; Smith and

Hindley, 1978; Whatley *et al.*, 1976). Cooksey and Moore (1982a) found evidence that the second mechanism played a role in the biological control of crown gall, as a K84$^-$ (non-agrocin-producing) mutant of *A. radiobacter* K84 was effective in reducing infection of an agrocin-sensitive isolate of *A. tumefaciens* when applied 24 h before the pathogen. However, unlike *A. radiobacter* K84, it was ineffective when inoculated together with the pathogen. Both the K84$^-$ mutant and *A. radiobacter* K84 reduced infection by an agrocin 84-resistant pathogen when applied 24-30 h before the pathogen.

A very important finding was that when the control strains were co-inoculated with the pathogenic isolates, at all control strain:pathogen cell ratios *A. radiobacter* K84 was superior to *A. tumefaciens* D286 in respect of the degree of control of the pathogenic isolates achieved (Tables 27 and 28). There also appeared to be no discernable synergistic effect above the reaction obtained with *A. radiobacter* K84 alone when *A. radiobacter* K84 and *A. tumefaciens* D286 were applied to plants together. (Note that *A. radiobacter* K84 was not inhibited by the agrocin of *A. tumefaciens* D286 and *vice versa*). However, the variation in the reactions of different isolates suggest that under field conditions, with certain strains of *A. tumefaciens* biotype 1 and 2 pathogens dominant in the soils, *A. tumefaciens* D286 or a combination of *A. tumefaciens* D286 and *A. radiobacter* K84 might have an advantage over *A. radiobacter* K84 alone in controlling infections. A comparison of the results obtained with tobacco (Table 27) and datura (Table 28) showed no differential effects of host plant.

The overall data on the inhibitory effects of *A. radiobacter* K84 and *A. tumefaciens* D286 on the *A. tumefaciens* biotype 1 and 2 isolates (Table 29) supports the blockage-of-infection-site hypothesis postulated by Lippincott and Lippincott (1969) and Whatley *et al.* (1976). The main reason for this conclusion is that the isolates which were agrocin-susceptible or agrocin-resistant *in vitro* showed similar inhibition percentages *in vivo*. However, it is probable that in these experiments the large numbers of agrocinogenic cells applied to the wounds might have immediately saturated the infection sites, preventing their occupation by the pathogens, and masking possible agrocin effects. Also, the assumption that a pathogen was resistant on the basis of a negative reaction *in vitro* on the three media can be questioned, if one takes into

consideration the marked influence of medium composition on susceptibility of the pathogenic isolates to agrocin-producing strains *in vitro*. *Agrobacterium tumefaciens* D286 showed a tendency to prevent tumour formation by a slightly higher percentage of pathogens susceptible to agrocin D286 (*in vitro*) than by non-susceptible isolates (Table 29) which could be indicative of agrocin involvement.

Inhibition of *Agrobacterium tumefaciens* and Tumour Development by Chemical Agents

The present study has shown clearly the unreliability of three possible chemical agents for crown gall control, namely, D-glucosamine (Richardson *et al.*, 1976), extract of *H. perforatum* (Popova and Cikova, 1978) and Gallex (Schroth and Hildebrand, 1968). The D-glucosamine failed to inhibit *A. tumefaciens* isolates *in vitro* and did not appear to have any effect on the test plant stem tumours, in contrast to its effect on the tumours on bean leaves as studied by Richardson *et al.* (1976). No effect on *Agrobacterium* isolates *in vitro* nor on test plant tumours was also observed with the *H. perforatum* extract and Gallex solution tested, notwithstanding the reports of Popova and Cikova (1978) and Schroth and Hildebrand (1968) that these chemical solutions inhibited pathogenic agrobacteria and tumour formation on various hosts or eradicated the tumorous material. For chemical treatment of tumours to be effective, the inhibitory chemical will have to either differentiate T-DNA-containing cells from normal plant cells, thereby selectively killing the tumour cells, or selectively destroy the pathogenic bacterium before the transplantation of the T-DNA. Chemicals which could be effective by suppressing the genetic dominance of the T-DNA might also await discovery.

Heat Sensitivity of *Agrobacterium tumefaciens* and Host Plant (Peach) Seedlings

In the present study, the most severe heat-treatment of 60°C for 80 min. was not effective in destroying the *A. tumefaciens* bacteria. Therefore, it appears that the ordinary heat-treatment of vines, potato tubers, cotton, budwood and dormant trees of various species (50°C for 15 min) for the control of viruses, some bacteria, certain fungi, mycoplasmas and rickettsias (Raychauduri and Verma, 1977; Von Broembsen and Marais, 1978) would not destroy the *A.*

tumefaciens bacteria. Also, the *A. tumefaciens* bacteria survived treatments (temperatures of at least 60°C for 80 min) that would irreversibly damage the host plants, (as indicated by the effect of heating peach trees for only 15 min at temperatures to 60°C) indicating that more severe heat treatments cannot be considered for crown gall control on peaches and probably also other plant material.

SUMMARY

1. This study was undertaken as a countrywide survey of the crown gall disease in the RSA, including strains of the pathogen (*Agrobacterium tumefaciens*) and the host plants affected by the disease. The susceptibility of the *A. tumefaciens* strains to biological control by two bacteriocinogenic agrobacteria (*Agrobacterium radiobacter* K84 and *A. tumefaciens* D286) was an important aspect of the investigation.

2. Pathogenic *A. tumefaciens* bacteria were found throughout the RSA on various host plants, including agriculturally important stonefruits, grapevines and chrysanthemums. Crown gall on fruit trees was found throughout the country, but on grapevines occurred mainly in the proximity of irrigation rivers outside the Western Cape region.

3. Young and fresh tumorous tissue yielded the causative bacteria with the highest frequency. Some tumorous tissue, especially old tumours, failed to yield the bacteria, probably because plant cells containing their T-DNA can continue producing tumourous tissue in the absence of the crown gall bacteria.

4. The best overall medium for the isolation of *A. tumefaciens* from tumours was nutrient agar (NA). It yielded the pathogenic bacterium more often than several selective media. The grafting of fresh parts of tumours onto an indicator host plant to produce tumour material suitable for plating was a good isolation method. The method also permitted a rapid presumptive identification of the pathogen prior to its isolation.

5. Isolation of *A. tumefaciens* from soils was achieved using selective media for the different biotypes. Non-selective NA plates were too rapidly overgrown by other bacteria. The crown gall pathogen was detected more frequently in close proximity to susceptible plant roots and crowns than in soil farther from the plants. The pathogen was obtained more often from sandy than from clay soils.

6. All South African *A. tumefaciens* isolates could be grouped into the traditional three biotypes by the standard tests. Of 65 isolates, 32 were biotype 1, 25 biotype 2 and eight biotype 3.

7. For standardizing *Agrobacterium* cell suspensions for plant inoculations and serological studies, colorimetric readings of absorbance of cell suspensions were calibrated against cell concentration by dilution plate count and a standard graph constructed from the regression equation.

8. Tobacco and datura seedlings were evaluated as better experimental indicator plants than broadbeans, tomatoes, sunflowers and carrot slices for tests of tumour formation by biotype 1 and 2 isolates. For biotype 3 isolates, Jacquez grapevine seedlings were better than a range of grapevine rootstocks. Seedlings of tobacco, but not datura or sunflower, were also suitable.

9. Serotyping of the *Agrobacterium* isolates showed them to be highly heterogenous. Wasserman tube agglutination tests with three antisera and washed cells showed seven different O- and four H-serogroups, while with well-washed cells and seven antisera, 18 different O-serogroups were detected. It appeared that the surface layer of the minimally washed cells masked deeper antigens which were exposed when the cells were well washed and certain outer components, including superficial antigens, removed.

Ouchterlony immunodiffusion reactions of phenol-treated cell suspensions explained some of the titre differences observed in the agglutination tests by indicating whether they were based on identity or partial identity of homologous and heterologous antigens reacting with a specific antiserum. Results obtained with phenol-treated minimally washed cells, but not well washed cells, in the Ouchterlony tests corresponded well with those obtained in the agglutination tests, possibly because of effective release and diffusion especially of the most superficial antigens in the Ouchterlony tests. The biotype 3 isolates produced no precipitin bands in the agar, but the homologous antisera appeared to precipitate the antigens in the antigen wells. Antigens which

provided very clear precipitin bands were released from the biotype 3 cells by the 'Boivin' extraction method.

An enzyme-linked immunosorbent assay (ELISA) method proved less sensitive for the demonstration of serological relationships than the agglutination and immunodiffusion techniques. In some cases ELISA showed different main antigenic affinities than those shown by the other two techniques. The threshold value of the ELISA method for a satisfactory reaction was 10^5-10^7 bacterial cells/ml.

From the various serological tests, 23 different serogroups were recognized according to their main O-antigenic affinities. Most serogroups contained bacteria of a specific biotype, but some contained two or three biotypes. Each biotype was represented by several serogroups. Some serogroups (8) were restricted to a specific biotype, host plant genus and geographical area, but others (15) had wider host ranges and/or distributions. The serological complexity of *A. tumefaciens* complicates serotyping and makes it unsuitable for easy diagnosis of biotypes and pathotypes of the crown gall organism.

10. Studies of the inhibition of *A. tumefaciens* isolates by *A. radiobacter* K84 and the bacteriocin-producing non-pathogenic *A. tumefaciens* D286 *in vitro*, indicated that three test media could show different sensitivity responses of a pathogen to the agrocinogenic strains. However, on at least one of the three test media, 63% and 68% of the biotype 1 and 2 isolates, respectively, were sensitive to agrocin 84, and 94% and 12%, respectively, to the bacteriocin of strain D286. The D286 bacteriocin thus inhibited certain biotype 1 isolates not inhibited by agrocin K84 but was mainly ineffective against the biotype 2 isolates. No biotype 3 isolate was inhibited by either bacteriocinogenic strain. Patterns of inhibition (all isolates) showed no correlation with serogroups.

Bacteriocin-resistant colonies developing inside inhibition zones on the test media were found to have lost their pathogenicity, producing no tumours when inoculated onto suitable host plants.

11. The *in vivo* inhibition (biological control) of the *A. tumefaciens* isolates by *A. tumefaciens* K84 and *A. tumefaciens* D286 was also studied. The highest percentages of control (>97%) were observed on the indicator plants tobacco and datura when the control strain was applied to wounds 24-30 h before the pathogenic isolate, or when the agrocin producer(s) and pathogen were applied simultaneously at a ratio of 10:1 or 3:1. Inhibition of tumour formation decreased progressively as the cell ratio of agrocin producer:pathogen decreased from 10:1 to 3:1 to 1:1. *Agrobacterium radiobacter* K84 was usually superior to *A. tumefaciens* D286 in respect of the percentage of pathogens controlled. No obvious synergistic effects were evident when the two bacteriocinogenic strains were applied together. However, the reactions of various isolates suggest that under field conditions, with certain *A. tumefaciens* biotype 1 and 2 isolates present in the soil, *A. tumefaciens* D286 or a combination of the two bacteriocinogenic strains might have an advantage over *A. radiobacter* K84 alone in controlling crown gall infections.

More isolates were inhibited *in vivo* than were susceptible to the bacteriocins in the *in vitro* studies. Furthermore, isolates which were bacteriocin-resistant *in vitro* showed similar percentages of inhibition *in vivo* to isolates that were bacteriocin-susceptible *in vitro*. These results support the blockage-of-infection-site hypothesis of the mechanism of inhibition, possibly combined with bacteriocin action in the case of sensitive bacteria.

12. None of three chemical agents (D-glucosamine, extract of the weed *Hypericum perforatum* and Gallex) was effective in inhibiting *A. tumefaciens* or the development of crown galls.

13. Heat treatment of *A. tumefaciens* at 60^{o}C for 80 min, which was too severe for peach seedlings, was inadequate to destroy the bacteria.

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APPENDIX

Biological Control Index (BCI) Values for Control of *A. tumefaciens* Tumour Formation on Tobacco and Datura by *A. tumefaciens* D286 and *A. radiobacter* K84

Treatments (with codes for column headings in Appendix Tables 1 and 2):

A - D286 and after 24 h the pathogen

B - K84 and after 24 h the pathogen

C - Pathogen and after 24 h D286

D - Pathogen and after 24 h K84

E - D286 and pathogen in a 1:1 ratio

F - K84 and pathogen in a 1:1 ratio

G - D286 and pathogen in a 3:1 ratio

H - K84 and pathogen in a 3:1 ratio

I - D286 and pathogen in a 1:3 ratio

J - K84 and pathogen in a 1:3 ratio

K - D286 and pathogen in a 10:1 ratio

L - K84 and pathogen in a 10:1 ratio

M - D286 and pathogen in a 1:10 ratio

N - K84 and pathogen in a 1:10 ratio

O - D286/K84 combination and pathogen in a 1:1 ratio

P - D286/K84 combination and pathogen in a 3:1 ratio

Q - D286/K84 combination and pathogen in a 1:3 ratio

R - D286/K84 combination and pathogen in a 10:1 ratio

S - D286/K84 combination and pathogen in a 1:10 ratio

T - Pathogen Control

Appendix Table 1. Biological control index values on tobacco plants

Pathogen	Treatments A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
B6	45.6	99.0	9.3	9.6	37.4	22.5	99.0	73.4	15.8	28.7	99.0	99.0	20.1	22.8	20.1	69.6	15.1	99.0	9.2	7.7
K17	99.0	99.0	33.3	9.1	79.3	19.5	99.0	99.0	45.9	81.9	99.0	99.0	54.9	74.4	99.0	99.0	99.0	99.0	52.5	27.0
K21	99.0	99.0	50.3	25.3	32.0	99.0	99.0	99.0	42.9	34.0	99.0	99.0	26.1	28.2	99.0	99.0	31.2	99.0	52.0	21.3
K28	99.0	99.0	11.3	55.1	99.0	99.0	95.2	99.0	43.6	99.0	99.0	99.0	42.2	19.5	99.0	99.0	29.2	99.0	25.8	23.3
K30	99.0	99.0	45.2	31.7	31.6	99.0	99.0	99.0	7.0	99.0	99.0	99.0	21.6	34.2	99.0	99.0	25.0	99.0	29.3	11.5
$N21_3$	99.0	99.0	33.6	22.3	46.2	99.0	36.7	93.7	36.1	86.7	12.9	62.9	41.1	40.0	71.7	72.6	99.0	99.0	36.9	13.2
$N21_4$	49.6	99.0	31.2	16.9	34.6	22.7	25.5	99.0	25.5	39.2	67.6	99.0	24.5	15.5	99.0	99.0	42.0	99.0	26.0	11.6
N37g	99.0	99.0	66.9	99.0	99.0	99.0	99.0	99.0	13.4	33.8	99.0	99.0	8.7	11.7	99.0	99.0	16.7	99.0	7.8	7.2
$N37_6$	99.0	99.0	25.6	22.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	61.3	55.1	99.0	99.0	99.0	99.0	37.2	14.4
$N37_{11}$	99.0	99.0	34.1	71.2	99.0	99.0	99.0	99.0	89.0	87.5	99.0	99.0	22.0	22.3	99.0	99.0	99.0	99.0	23.7	21.3
N51g	99.0	99.0	46.3	31.0	99.0	99.0	99.0	99.0	20.3	51.7	99.0	99.0	38.1	50.5	99.0	99.0	99.0	99.0	99.0	12.5
$N51_6$	99.0	99.0	50.7	21.7	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	79.6	16.0
$N52_3$	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	34.1	99.0	99.0	99.0	34.3	26.1	99.0	99.0	99.0	99.0	32.9	19.1
$N52_5$	99.0	99.0	64.6	31.1	72.9	99.0	99.0	99.0	14.8	10.2	99.0	99.0	13.1	9.4	37.0	99.0	22.9	99.0	7.8	12.8
$N57_2$	99.0	99.0	98.0	20.1	99.0	99.0	99.0	99.0	28.4	19.2	99.0	99.0	65.7	24.4	99.0	99.0	20.7	99.0	16.6	22.2
TT9	99.0	99.0	99.0	55.3	99.0	99.0	99.0	99.0	80.0	99.0	99.0	99.0	24.7	45.5	99.0	99.0	99.0	99.0	35.6	12.6
T-37	99.0	99.0	44.0	6.5	99.0	12.5	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	14.1
450	99.0	99.0	49.3	31.2	24.6	43.5	37.9	28.0	10.6	21.7	26.4	99.0	10.8	12.9	82.8	99.0	13.6	99.0	16.6	12.3
57	99.0	99.0	43.6	25.0	65.8	99.0	72.3	99.0	45.0	38.1	99.0	99.0	31.4	17.0	99.0	99.0	19.4	99.0	27.6	9.4
143	99.0	99.0	27.8	28.4	24.5	21.0	99.0	99.0	19.8	71.3	99.0	99.0	11.3	30.5	99.0	99.0	13.4	99.0	10.0	8.7
172(a)	99.0	99.0	18.7	12.1	19.6	99.0	99.0	99.0	99.0	45.3	99.0	99.0	49.8	21.2	99.0	99.0	99.0	99.0	44.7	35.0
172(b)	99.0	99.0	20.7	11.3	32.3	99.0	99.0	99.0	27.4	17.9	99.0	99.0	16.0	23.8	99.0	99.0	99.0	99.0	39.9	14.2
196	99.0	99.0	43.0	18.8	99.0	99.0	99.0	99.0	99.0	55.8	99.0	99.0	15.9	34.0	99.0	99.0	18.7	99.0	48.0	10.3
925	99.0	99.0	6.5	20.3	26.2	11.2	52.0	16.8	17.8	38.3	99.0	99.0	37.2	15.8	14.7	99.0	22.9	34.1	10.4	9.6
1465	99.0	99.0	11.2	32.5	19.7	14.8	17.8	47.7	12.6	11.7	21.4	24.0	8.3	8.9	12.8	57.0	22.2	88.7	9.0	8.8
1477	99.0	99.0	21.1	15.5	27.6	53.8	99.0	99.0	12.6	23.7	99.0	99.0	16.7	11.7	99.0	99.0	99.0	99.0	19.3	6.2
1887	99.0	99.0	65.5	49.6	99.0	99.0	99.0	99.0	28.4	99.0	99.0	99.0	22.1	21.0	99.0	99.0	99.0	99.0	18.9	15.3
1G95	99.0	99.0	28.3	26.9	99.0	41.8	99.0	99.0	29.3	31.2	99.0	99.0	30.8	21.3	99.0	99.0	21.4	99.0	39.4	9.6
2080	99.0	99.0	31.8	35.2	76.1	50.4	45.3	67.7	17.2	29.6	99.0	99.0	13.8	14.2	99.0	99.0	24.5	99.0	15.5	15.6
2086A	99.0	99.0	17.1	61.9	39.7	99.0	99.0	99.0	22.2	99.0	99.0	99.0	18.8	28.9	99.0	99.0	99.0	99.0	29.2	10.8
2153	99.0	99.0	13.1	18.6	99.0	99.0	99.0	99.0	99.0	29.3	99.0	99.0	22.0	25.0	99.0	99.0	55.5	99.0	10.9	9.6
4452	99.0	99.0	22.9	12.4	43.3	27.5	36.6	30.0	21.4	19.0	72.5	42.6	15.0	10.9	40.3	99.0	16.9	99.0	8.8	8.2
A1	99.0	99.0	60.2	10.0	99.0	99.0	99.0	99.0	19.6	99.0	99.0	99.0	10.7	48.5	49.0	99.0	99.0	99.0	14.9	11.6
A5	99.0	99.0	39.8	36.3	57.7	99.0	99.0	99.0	76.4	51.0	99.0	99.0	28.9	33.4	99.0	99.0	28.5	99.0	31.4	20.8
A6	99.0	99.0	21.7	72.7	22.3	66.2	59.3	99.0	31.4	99.0	99.0	99.0	35.5	24.3	99.0	99.0	99.0	99.0	16.7	13.5
C5	99.0	99.0	16.5	40.1	28.9	27.6	99.0	99.0	30.8	99.0	99.0	99.0	23.6	29.2	99.0	99.0	99.0	99.0	18.6	14.9
B3	99.0	99.0	38.8	35.2	9.1	57.6	11.5	89.9	6.7	7.4	99.0	99.0	4.3	6.2	10.7	21.9	4.6	99.0	3.5	4.4
D6	99.0	99.0	9.8	9.2	58.9	99.0	99.0	99.0	13.5	31.4	99.0	99.0	30.6	7.5	93.6	99.0	42.5	99.0	9.1	9.8
D8	99.0	99.0	42.4	49.9	99.0	11.2	88.9	99.0	21.9	30.8	99.0	99.0	9.0	10.9	99.0	99.0	9.9	99.0	11.1	10.2
D10	99.0	99.0	29.3	42.7	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	44.9	99.0	99.0	99.0	8.7	99.0	10.1	8.8
127	99.0	99.0	94.8	30.2	99.0	99.0	99.0	99.0	32.6	99.0	99.0	99.0	17.1	99.0	99.0	99.0	99.0	99.0	22.0	18.4
Z8	99.0	99.0	55.2	36.5	55.8	60.6	77.6	63.9	99.0	97.5	90.8	87.3	66.9	47.9	32.5	96.7	45.1	76.0	54.8	66.7
Z12	99.0	99.0	52.5	33.6	18.4	46.6	99.0	99.0	22.4	99.0	99.0	99.0	24.3	22.6	99.0	99.0	23.0	99.0	22.5	20.5
Z13	99.0	99.0	75.5	34.3	99.0	99.0	99.0	99.0	55.8	52.6	99.0	99.0	29.2	33.5	84.7	99.0	28.0	99.0	27.0	15.9
Z36	99.0	99.0	99.0	56.0	34.6	69.6	99.0	99.0	15.9	9.9	99.0	99.0	7.0	8.6	99.0	99.0	12.9	99.0	6.1	6.3
39g	99.0	99.0	40.3	46.6	76.3	99.0	99.0	99.0	24.1	99.0	99.0	99.0	14.4	64.3	99.0	99.0	99.0	99.0	21.0	18.9
39i	99.0	99.0	21.2	38.6	37.4	55.2	53.3	99.0	28.7	99.0	99.0	99.0	31.4	99.0	99.0	99.0	99.0	99.0	32.5	30.1
39m	99.0	99.0	35.7	20.8	99.0	13.1	99.0	99.0	22.5	16.9	99.0	99.0	8.9	27.7	62.7	99.0	12.2	99.0	5.0	5.4
39n	99.0	99.0	21.0	17.7	21.8	48.1	35.7	99.0	19.5	99.0	99.0	99.0	12.4	22.5	99.0	99.0	73.2	99.0	17.8	11.7
41B	99.0	99.0	61.4	25.4	51.0	38.5	43.7	99.0	65.2	99.0	81.0	99.0	16.2	24.8	99.0	99.0	72.0	99.0	15.8	14.8
46	99.0	99.0	54.2	81.4	38.4	35.6	33.5	99.0	30.5	99.0	99.0	99.0	54.9	82.6	32.1	95.1	33.1	99.0	34.9	20.2
47	99.0	99.0	5.2	10.3	27.6	99.0	31.5	99.0	7.3	6.6	99.0	99.0	5.0	11.4	36.0	86.3	99.0	99.0	6.0	4.7
48	99.0	99.0	69.9	99.0	42.1	93.2	33.4	99.0	46.1	99.0	99.0	99.0	10.9	9.3	31.8	99.0	8.6	99.0	16.0	13.1
49	88.7	99.0	47.5	29.5	26.4	64.0	30.5	99.0	16.1	17.7	99.0	99.0	13.6	12.0	34.8	99.0	12.0	99.0	8.6	8.6
70	99.0	99.0	18.4	61.0	65.2	99.0	99.0	99.0	48.4	99.0	99.0	99.0	76.6	50.3	99.0	99.0	41.6	99.0	52.2	10.9
71	99.0	99.0	27.0	20.5	99.0	56.8	99.0	99.0	35.6	47.9	99.0	99.0	9.5	9.3	99.0	99.0	22.9	99.0	8.2	8.1
73	99.0	80.0	63.7	37.0	99.0	42.8	87.3	97.8	10.5	24.6	99.0	99.0	9.8	7.4	99.0	99.0	38.0	99.0	16.3	11.9
2077	99.0	99.0	80.0	89.4	99.0	99.0	99.0	99.0	50.3	30.2	99.0	99.0	9.4	16.7	99.0	99.0	99.0	99.0	48.0	5.8
W7	99.0	99.0	26.5	80.4	99.0	99.0	99.0	81.8	73.0	71.9	99.0	99.0	88.0	99.0	99.0	99.0	42.0	99.0	92.0	82.1
W8	99.0	99.0	28.8	21.2	24.9	79.9	99.0	99.0	24.2	85.4	99.0	99.0	8.7	26.8	99.0	99.0	29.1	99.0	23.6	17.9
305	99.0	99.0	38.8	60.3	99.0	29.8	42.9	33.8	20.8	44.8	9.3	41.8	30.5	33.2	24.9	66.2	33.6	99.0	16.4	21.0
1771	99.0	99.0	99.0	81.5	99.0	99.0	99.0	99.0	30.7	31.4	99.0	99.0	12.6	34.8	39.0	48.5	40.1	86.5	42.6	29.1
2160	99.0	99.0	21.8	19.7	99.0	99.0	99.0	99.0	99.0	37.2	99.0	99.0	21.8	18.6	99.0	99.0	99.0	99.0	29.1	22.1
2164	99.0	99.0	99.0	99.0	56.6	99.0	99.0	99.0	38.8	75.8	99.0	99.0	28.1	59.8	99.0	99.0	86.8	99.0	61.8	32.2
2221	99.0	99.0	20.0	32.5	32.6	28.2	32.6	39.6	50.2	97.6	56.1	46.7	22.9	49.8	99.0	99.0	74.2	57.6	32.4	23.9

Appendix Table 2. Biological control index values on datura plants

Pathogen	Treatments																			
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
B6	72.2	99.0	6.2	9.3	51.0	36.8	61.4	99.0	12.3	12.2	88.4	99.0	10.5	11.3	41.0	46.2	16.2	99.0	11.2	8.1
E17	99.0	99.0	24.0	8.6	97.4	81.3	92.1	99.0	85.6	97.0	99.0	99.0	72.3	68.7	99.0	99.0	99.0	99.0	66.1	21.5
E21	99.0	99.0	79.6	98.3	99.0	99.0	99.0	99.0	28.8	26.9	99.0	99.0	26.3	29.2	99.0	99.0	30.2	99.0	28.4	14.1
E28	99.0	99.0	59.8	26.7	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	56.5	53.5	99.0	99.0	99.0	99.0	22.4	21.4
E30	99.0	99.0	63.6	29.5	23.4	38.9	99.0	99.0	11.2	99.0	99.0	99.0	40.4	36.7	99.0	99.0	50.6	99.0	47.4	12.4
M21$_3$	53.7	99.0	24.0	25.0	52.4	99.0	74.0	99.0	16.8	77.0	12.7	99.0	19.3	28.2	99.0	99.0	62.5	99.0	19.5	13.5
M21$_4$	77.8	99.0	29.5	17.7	33.7	23.6	21.3	99.0	16.1	61.2	23.5	99.0	13.7	33.7	64.2	99.0	42.3	99.0	41.6	24.7
M37$_5$	99.0	99.0	17.4	22.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	38.8	58.5	99.0	99.0	99.0	99.0	99.0	12.6
M37$_9$	99.0	99.0	23.6	99.0	99.0	99.0	99.0	99.0	11.7	30.5	99.0	99.0	10.3	8.4	99.0	99.0	9.1	99.0	6.4	6.5
M37$_{11}$	99.0	99.0	21.2	26.6	99.0	99.0	99.0	99.0	59.7	31.5	99.0	99.0	19.2	23.1	99.0	99.0	99.0	99.0	19.9	20.3
M51$_6$	99.0	99.0	18.6	30.8	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	32.0
M51$_9$	99.0	99.0	50.6	17.6	99.0	99.0	99.0	99.0	18.0	26.9	99.0	99.0	99.0	32.4	99.0	99.0	99.0	99.0	99.0	22.1
M52$_3$	99.0	99.0	36.5	6.7	99.0	99.0	99.0	99.0	32.1	99.0	99.0	99.0	21.8	85.4	99.0	99.0	99.0	99.0	15.6	15.4
M52$_5$	99.0	99.0	39.9	26.5	92.9	99.0	99.0	99.0	11.8	10.5	99.0	99.0	9.7	10.1	41.2	99.0	12.7	99.0	10.5	10.6
M57$_2$	99.0	99.0	99.0	76.9	99.0	99.0	99.0	99.0	19.9	84.8	99.0	99.0	16.6	41.2	99.0	99.0	16.5	99.0	28.3	14.8
TT9	99.0	99.0	14.5	19.8	57.1	99.0	99.0	99.0	62.3	83.2	99.0	99.0	23.7	29.8	99.0	99.0	99.0	99.0	23.5	11.5
T-37	99.0	99.0	28.3	12.0	99.0	23.6	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	18.6
450	99.0	99.0	61.5	17.4	22.8	38.4	25.5	99.0	11.0	28.5	25.6	99.0	7.2	10.8	99.0	99.0	11.0	99.0	14.4	13.6
57	99.0	99.0	12.3	24.1	33.8	99.0	59.5	99.0	31.0	29.2	99.0	99.0	11.9	11.2	99.0	99.0	29.2	99.0	9.8	10.8
143	99.0	99.0	12.1	42.0	43.9	38.9	99.0	99.0	15.4	10.4	99.0	99.0	8.4	60.4	99.0	99.0	13.8	99.0	7.4	8.2
172(a)	99.0	99.0	12.2	12.5	9.3	99.0	99.0	99.0	74.4	53.9	99.0	99.0	20.0	27.4	99.0	99.0	87.2	99.0	40.4	18.8
172(b)	99.0	99.0	11.5	9.9	9.6	58.1	99.0	99.0	21.7	40.1	99.0	99.0	8.6	15.7	99.0	99.0	53.5	99.0	31.0	13.9
198	99.0	99.0	48.9	10.9	32.3	99.0	99.0	99.0	47.8	46.7	99.0	99.0	15.9	53.3	99.0	99.0	52.2	99.0	55.2	26.6
925	99.0	99.0	9.4	13.3	20.8	13.7	70.6	99.0	14.4	11.6	88.8	99.0	22.6	11.4	33.0	99.0	11.0	99.0	9.7	8.6
1465	99.0	99.0	30.9	15.9	99.0	51.4	20.6	36.5	11.2	34.8	24.5	99.0	6.8	18.7	79.3	37.1	28.9	66.3	8.7	7.8
1477	99.0	99.0	11.6	8.0	47.6	19.8	23.7	99.0	13.4	59.4	23.8	99.0	14.0	15.0	99.0	99.0	99.0	99.0	13.6	7.4
1887	99.0	99.0	31.8	41.3	99.0	99.0	99.0	99.0	26.7	91.4	99.0	99.0	25.7	23.6	99.0	99.0	99.0	99.0	23.4	14.1
1895	99.0	99.0	17.9	23.9	90.0	99.0	72.0	99.0	19.8	14.2	99.0	99.0	12.2	9.1	99.0	99.0	11.8	99.0	7.7	6.0
2080	99.0	99.0	86.4	30.8	99.0	48.0	37.6	55.8	48.5	37.0	99.0	99.0	17.9	18.8	99.0	99.0	35.8	99.0	12.5	14.6
2086A	99.0	99.0	17.2	10.1	28.5	99.0	52.1	99.0	30.7	99.0	99.0	99.0	19.7	26.0	99.0	99.0	99.0	99.0	27.2	7.2
2153	99.0	99.0	8.6	23.3	21.9	99.0	74.8	99.0	19.9	14.1	99.0	99.0	16.0	9.0	99.0	99.0	10.8	99.0	10.3	9.6
4452	99.0	99.0	22.6	14.1	19.2	17.2	25.9	33.1	26.6	22.1	72.1	59.3	13.7	8.6	20.7	99.0	20.0	99.0	8.4	8.2
A1	99.0	99.0	11.4	8.9	99.0	99.0	99.0	99.0	12.5	99.0	99.0	99.0	10.7	28.6	49.9	99.0	73.0	99.0	15.7	14.1
A5	99.0	99.0	53.0	31.5	46.4	99.0	35.8	99.0	46.3	39.3	99.0	99.0	26.0	30.6	99.0	99.0	27.5	99.0	27.2	13.2
A6	99.0	99.0	27.5	99.0	18.0	37.7	46.5	99.0	13.8	69.9	99.0	99.0	11.5	21.8	99.0	99.0	99.0	99.0	15.6	10.1
C5	99.0	99.0	20.5	37.5	26.6	42.9	41.8	99.0	28.8	99.0	53.0	99.0	12.6	56.1	99.0	99.0	99.0	99.0	15.7	22.9
D3	99.0	99.0	29.6	48.5	13.0	30.5	17.4	82.9	6.7	3.1	99.0	99.0	4.2	3.1	11.4	13.5	3.7	80.9	4.7	3.7
D6	99.0	99.0	7.2	8.7	99.0	94.0	67.1	96.0	9.0	7.6	99.0	99.0	4.5	6.0	99.0	99.0	6.1	99.0	10.2	5.2
D8	99.0	55.4	11.9	11.7	8.6	99.0	84.8	99.0	7.6	6.0	99.0	99.0	5.5	7.5	99.0	99.0	5.1	99.0	4.6	5.7
D10	99.0	99.0	33.0	22.1	99.0	99.0	99.0	60.2	99.0	12.8	99.0	99.0	8.5	9.4	98.6	99.0	6.6	99.0	7.4	4.4
I27	99.0	99.0	83.6	21.0	71.2	99.0	99.0	99.0	9.4	99.0	99.0	99.0	8.7	99.0	99.0	99.0	99.0	99.0	28.8	8.6
Z8	99.0	99.0	39.7	32.9	16.6	42.7	16.2	55.5	96.8	66.0	24.9	86.6	42.7	63.7	30.2	54.1	87.9	99.0	40.1	24.3
Z12	35.4	45.1	7.7	41.4	16.1	32.9	42.5	99.0	25.9	57.0	99.0	99.0	25.3	29.5	97.4	99.0	23.1	99.0	25.0	27.2
Z13	99.0	99.0	68.0	26.6	99.0	99.0	99.0	99.0	23.4	38.1	99.0	99.0	26.7	35.9	99.0	99.0	32.2	99.0	24.1	18.9
Z36	99.0	99.0	20.8	11.1	49.1	80.6	87.9	99.0	10.0	7.8	99.0	99.0	5.6	9.1	54.1	99.0	7.9	99.0	9.1	7.0
39g	99.0	99.0	31.9	27.5	79.5	99.0	99.0	99.0	19.7	99.0	99.0	99.0	25.6	48.1	99.0	99.0	99.0	99.0	21.6	8.2
39i	99.0	99.0	25.4	22.1	45.2	32.5	26.3	99.0	13.3	99.0	99.0	99.0	21.0	99.0	99.0	99.0	99.0	99.0	31.3	9.5
39m	99.0	99.0	18.5	52.6	81.2	61.0	99.0	99.0	15.2	19.2	99.0	99.0	10.7	33.4	99.0	99.0	7.0	99.0	6.7	6.3
39n	99.0	99.0	40.5	17.2	99.0	99.0	79.8	99.0	11.2	99.0	99.0	99.0	11.1	12.8	99.0	99.0	23.6	99.0	23.2	7.5
41B	42.8	99.0	8.5	22.6	22.0	45.3	26.3	99.0	31.8	92.3	86.4	99.0	28.4	56.1	99.0	99.0	77.9	99.0	16.1	12.1
46	99.0	99.0	43.8	62.0	31.5	66.5	33.6	99.0	12.6	20.2	99.0	99.0	6.0	5.8	52.0	72.7	5.0	99.0	5.6	3.5
47	99.0	99.0	5.9	3.4	33.3	99.0	25.5	99.0	4.9	7.6	99.0	99.0	7.6	5.8	49.8	85.9	99.0	99.0	3.4	4.7
48	99.0	99.0	60.1	64.7	31.0	99.0	24.2	99.0	8.6	21.3	99.0	99.0	11.1	8.9	99.0	99.0	11.0	99.0	15.3	9.3
49	99.0	99.0	44.7	21.1	76.2	78.2	43.8	99.0	13.0	12.5	99.0	99.0	9.5	9.8	61.1	99.0	8.1	99.0	7.3	9.4
70	99.0	99.0	10.3	9.7	96.7	99.0	99.0	99.0	15.5	33.0	99.0	99.0	12.3	16.1	99.0	99.0	79.5	99.0	43.0	10.3
71	99.0	99.0	22.5	24.6	99.0	64.5	99.0	99.0	9.6	15.4	99.0	99.0	9.7	9.0	96.6	99.0	13.4	99.0	11.3	9.5
73	99.0	99.0	38.7	12.2	99.0	32.6	59.4	99.0	8.8	35.1	99.0	99.0	6.6	8.6	99.0	99.0	22.6	99.0	9.6	6.6
2077	99.0	99.0	11.9	54.9	99.0	99.0	99.0	99.0	21.2	47.9	99.0	99.0	12.2	55.1	99.0	99.0	99.0	99.0	52.3	10.0
V7	95.6	19.0	23.6	11.5	68.9	74.3	99.0	99.0	34.5	99.0	99.0	99.0	28.8	22.4	99.0	99.0	99.0	99.0	99.0	27.7
V8	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0
305	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0
1771	99.0	99.0	46.3	59.0	99.0	59.8	99.0	99.0	22.1	99.0	81.2	99.0	10.8	45.5	33.9	76.9	48.5	71.7	26.8	20.5
2160	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0
2164	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0
2221	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0